US009399776B2

(12) **United States Patent**
Friedrich et al.

(10) **Patent No.: US 9,399,776 B2**
(45) **Date of Patent: Jul. 26, 2016**

(54) ***CHLOROGLOEOPSIS* SP. HOST CELL FOR PRODUCING ETHANOL AND METHOD FOR PRODUCING ETHANOL USING THE SAME**

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(73) Assignee: **Algenol Biotech LLC**, Fort Myers, FL (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 4 days.

(21) Appl. No.: **14/839,359**

(22) Filed: **Aug. 28, 2015**

(65) **Prior Publication Data**

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Related U.S. Application Data

(63) Continuation of application No. PCT/EP2013/077496, filed on Dec. 19, 2013.

(51) **Int. Cl.**

C12N 1/13	(2006.01)
C12N 15/52	(2006.01)
C12N 15/74	(2006.01)
C12N 9/88	(2006.01)
C12N 9/04	(2006.01)

(52) **U.S. Cl.**

CPC ***C12N 15/52*** (2013.01); ***C12N 9/0006*** (2013.01); ***C12N 9/88*** (2013.01); ***C12N 15/74*** (2013.01); *C12Y 101/01001* (2013.01); *C12Y 401/01001* (2013.01)

(58) **Field of Classification Search**

None

See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

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Mitra et al. (1967), "On a new genus of the blue-green alga *Chlorogloeopsis* with remarks on the production of heterocysts in the alga," Phykos 5:106-114.

Mitra et al. (1950), "Two new algae from Indian soils," Ann. Bot. London. N.S. 14: 457-464.

Stucken et al. (2012), "Transformation and Conjugal Transfer of Foreign Genes into the Filamentous Multicellular Cyanobacteria (Subsection V) Fischerella and Chlorogloeopsis," Curr Microbiol., 65:552-560.

International Search Report and Written Opinion for PCT/EP2013/077496, dated Feb. 20, 2014 (9 pages).

Primary Examiner — Jim Ketter

(74) *Attorney, Agent, or Firm* — Lawrence B. Ebert; Suzanne G. Jepson; David J. Lorenz

(57) **ABSTRACT**

One embodiment of the invention provides a genetically enhanced *Chlorogloeopsis* sp. host cell comprising at least one first recombinant gene encoding a first protein for the production of ethanol under the transcriptional control of a first inducible promoter, having at least 85%, 90% or 95% sequence identity to an endogenous inducible promoter of the *Chlorogloeopsis* sp. host cell.

19 Claims, 33 Drawing Sheets

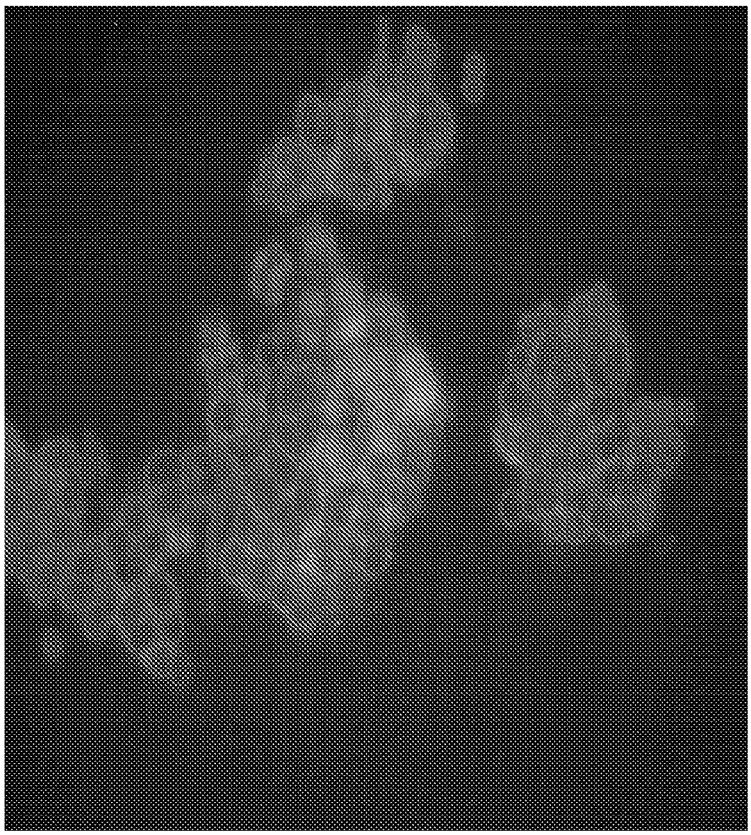


FIG. 1

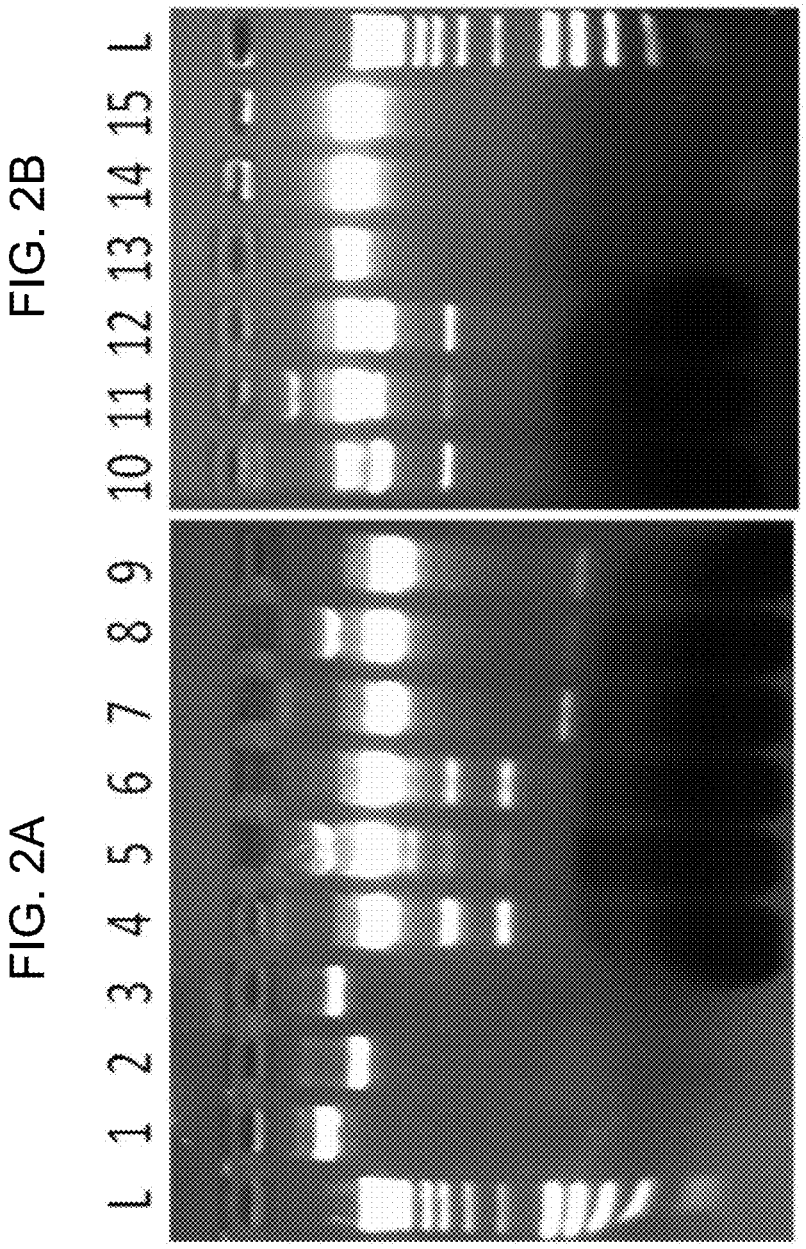


FIG. 2A

FIG. 2B

FIG. 3B

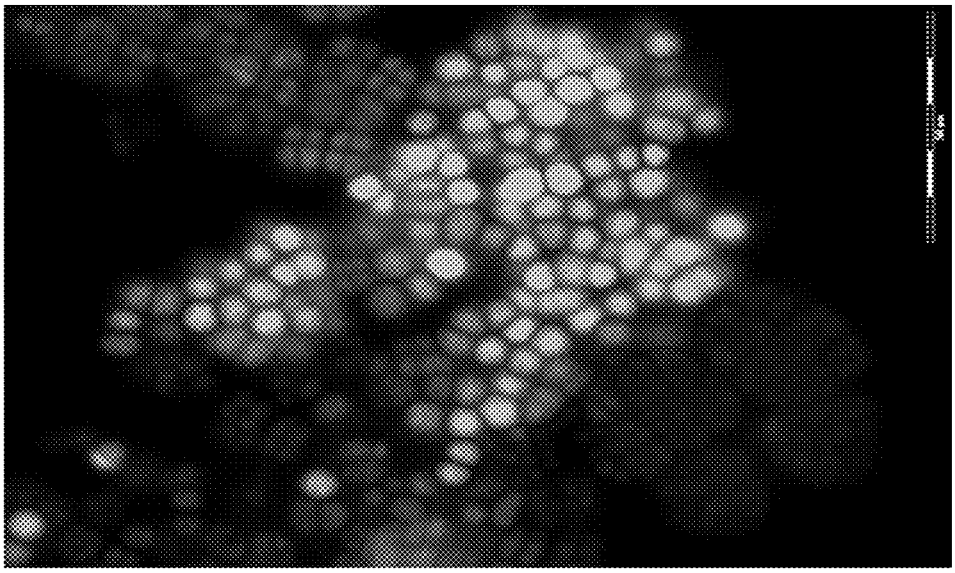
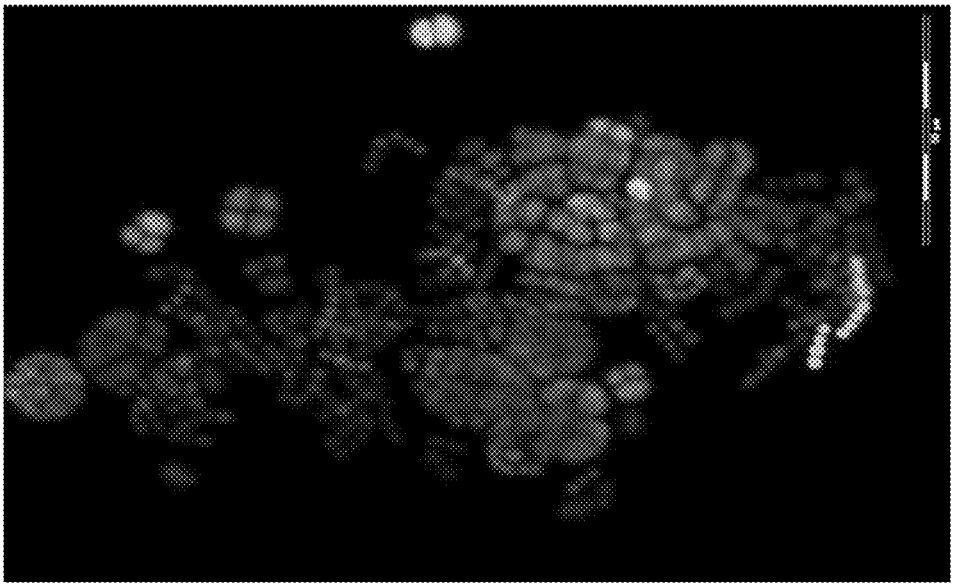


FIG. 3A



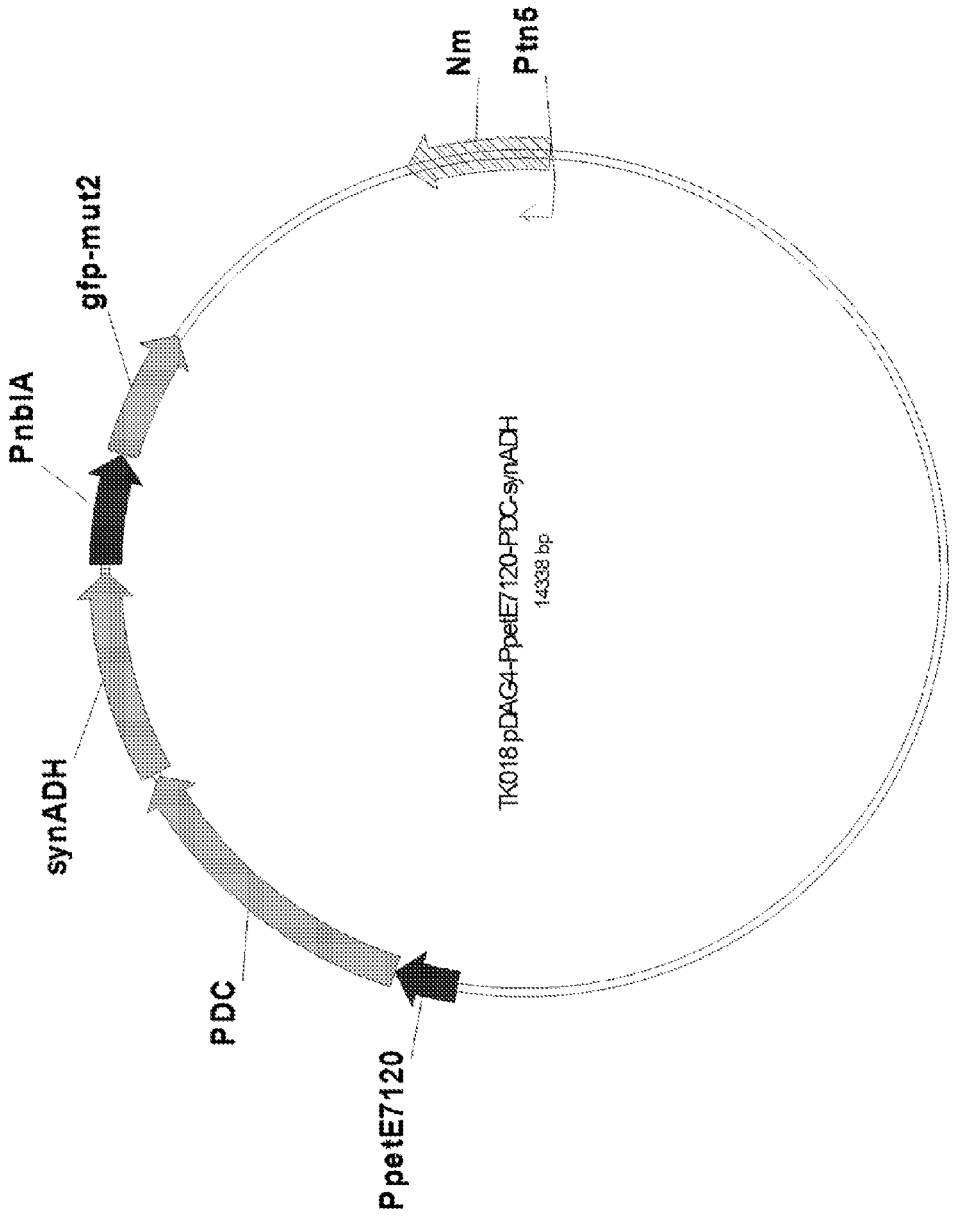
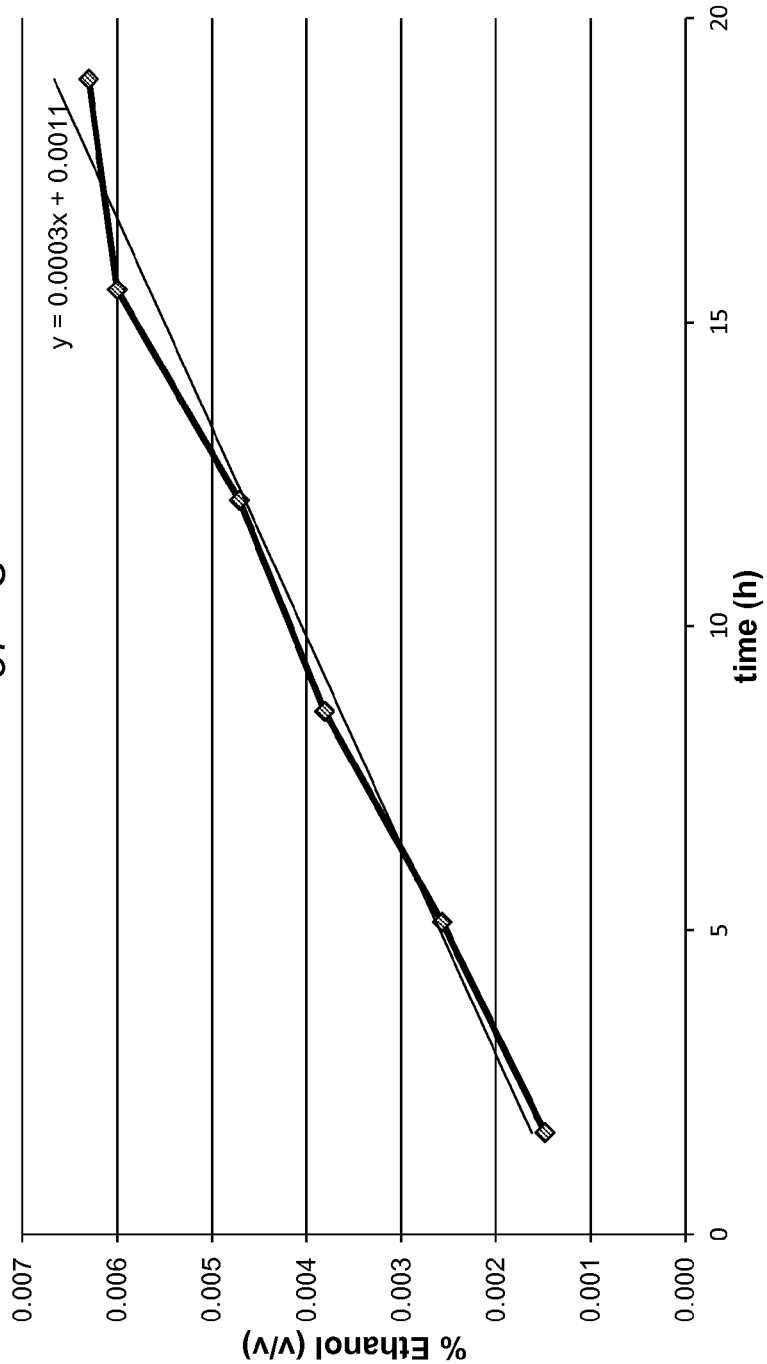

Nm
Ptn5
gfp-mut2
PnblA
synADH
PDC
PpetE7120
TK018 pDAG4-PpetE7120-PDC-synADH
14338 bp

FIG. 4A

FIG. 4B


Chlorogloeopsis sp. PCC 6912+ TK18 Ethanol production at 37° C
y = 0.0003x + 0.0011
% Ethanol (v/v)
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0.000
0
5
10
15
20
time (h)

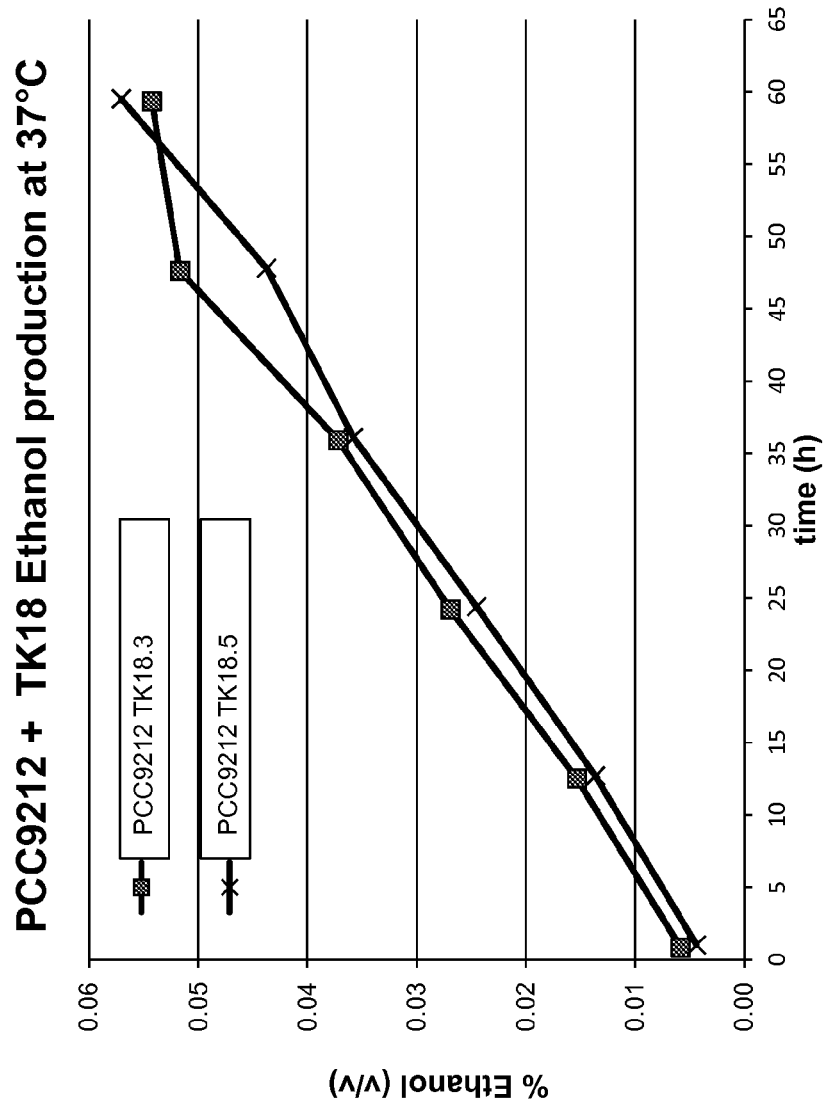

PCC9212 + TK18 Ethanol production at 37°C
PCC9212 TK18.3
PCC9212 TK18.5
% Ethanol (v/v)
0.00
0.01
0.02
0.03
0.04
0.05
0.06
time (h)
0
5
10
15
20
25
30
35
40
45
50
55
60
65

FIG. 4C

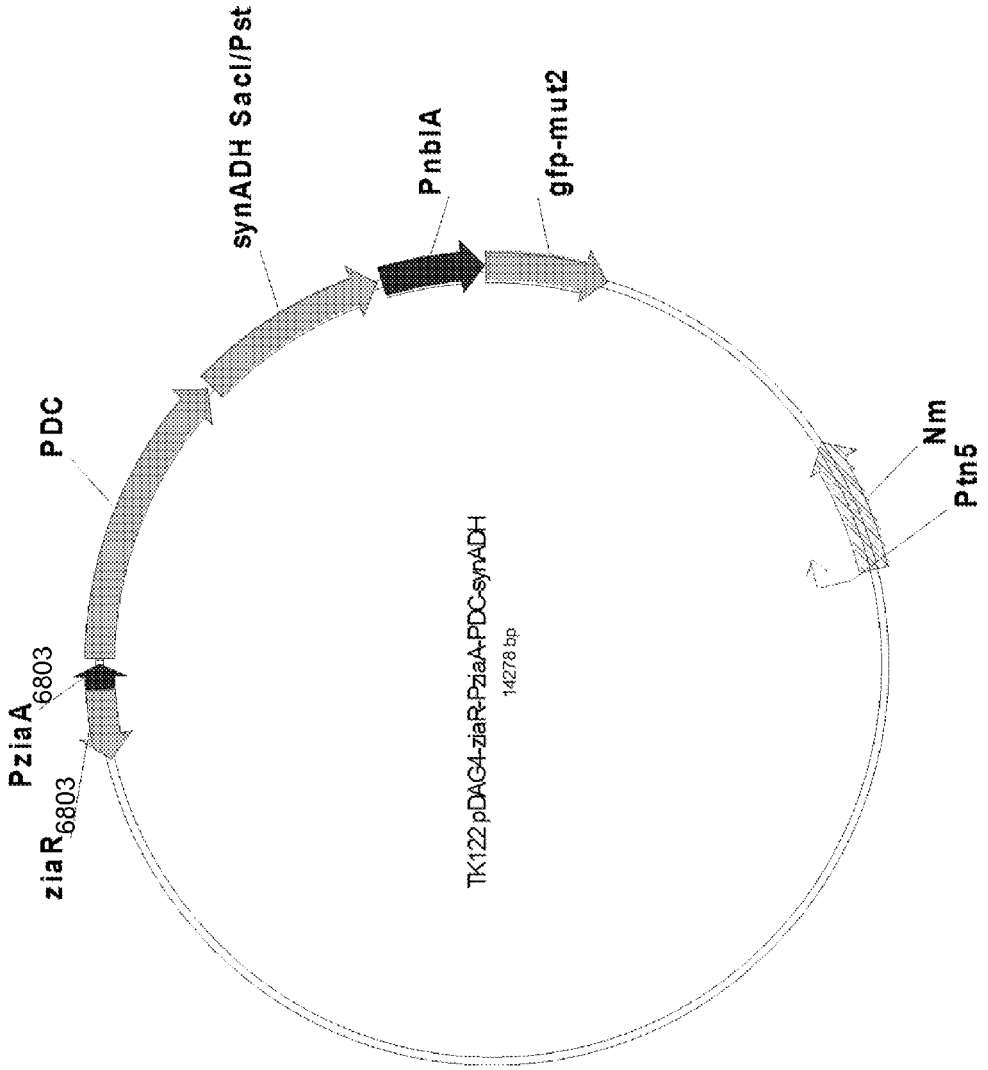

synADH SacI/Pst
PnblA
gfp-mut2
PDC
Nm
Ptn5
PziaA6803
ziaR6803
TK122 pDAG4-ziaR-PziaA-PDC-synADH
14278 bp

FIG. 5

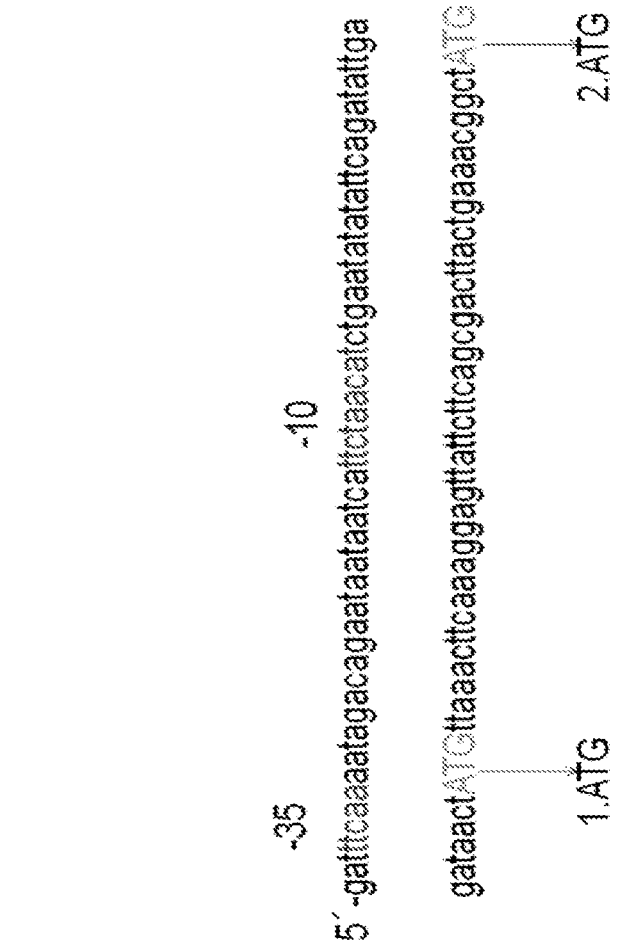

-35
-10
5′-gatttcaaaatagacagaataataatcatttctaacatctgaatatatattcagatattga
gataactATGttaaacttcaaaggagttattcttcagcgacttactgaaacggctATG
1.ATG
2.ATG

FIG. 6

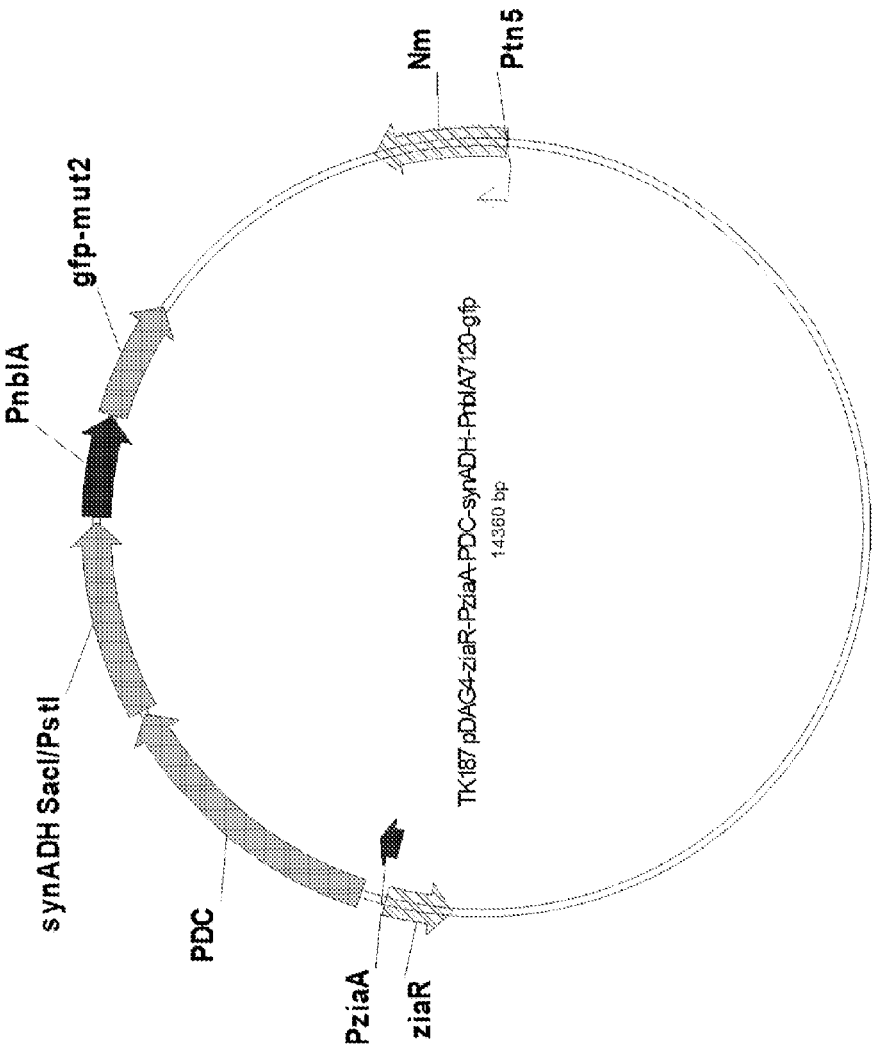

Nm
Ptn5
gfp-mut2
PnbiA
synADH SacI/PstI
PDC
PziaA
ziaR
TK187 pDAG4-ziaR-PziaA-PDC-synADH-PnbiA7120-gfp
14360 bp

FIG. 7A

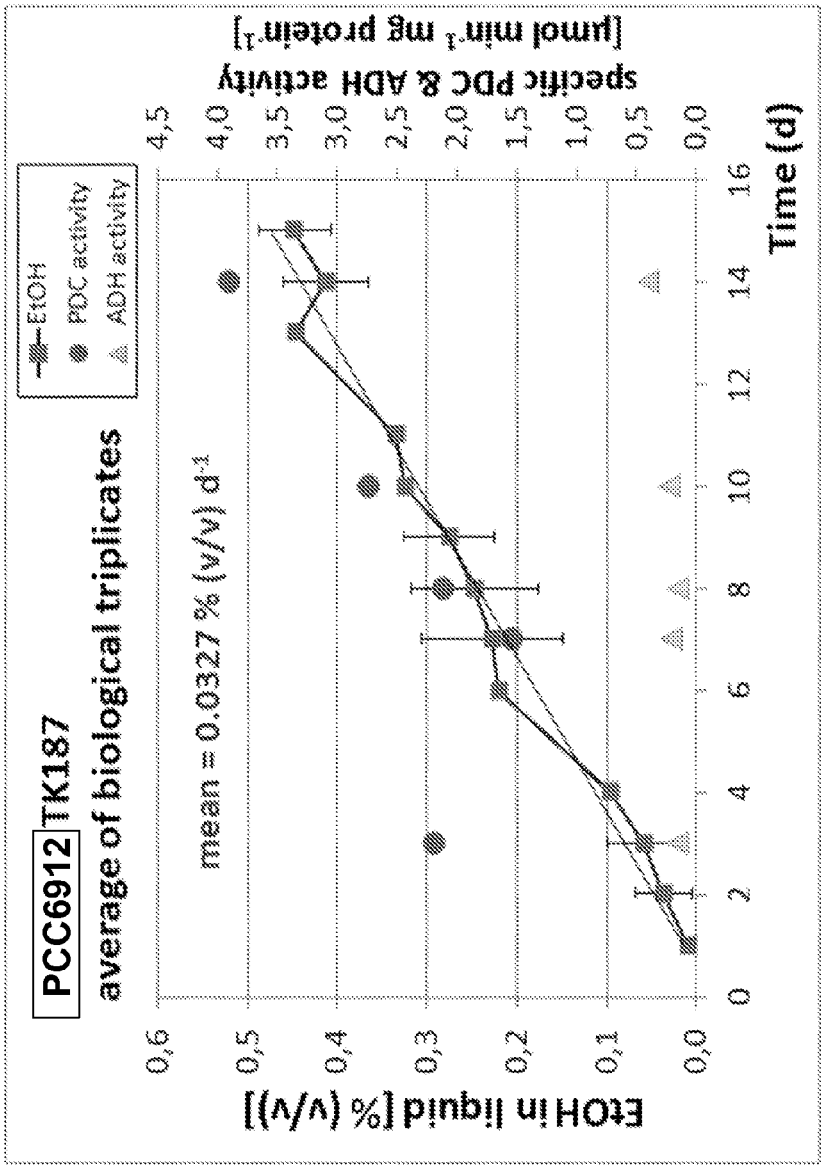

PCC6912 TK187
average of biological triplicates
mean = 0.0327 % (v/v) d-1
EtOH
PDC activity
ADH activity
EtOH in liquid [% (v/v)]
specific PDC & ADH activity
[µmol min-1 mg protein-1]
Time (d)
0,0
0,1
0,2
0,3
0,4
0,5
0,6
0,0
0,5
1,0
1,5
2,0
2,5
3,0
3,5
4,0
4,5
0
2
4
6
8
10
12
14
16

FIG. 7B

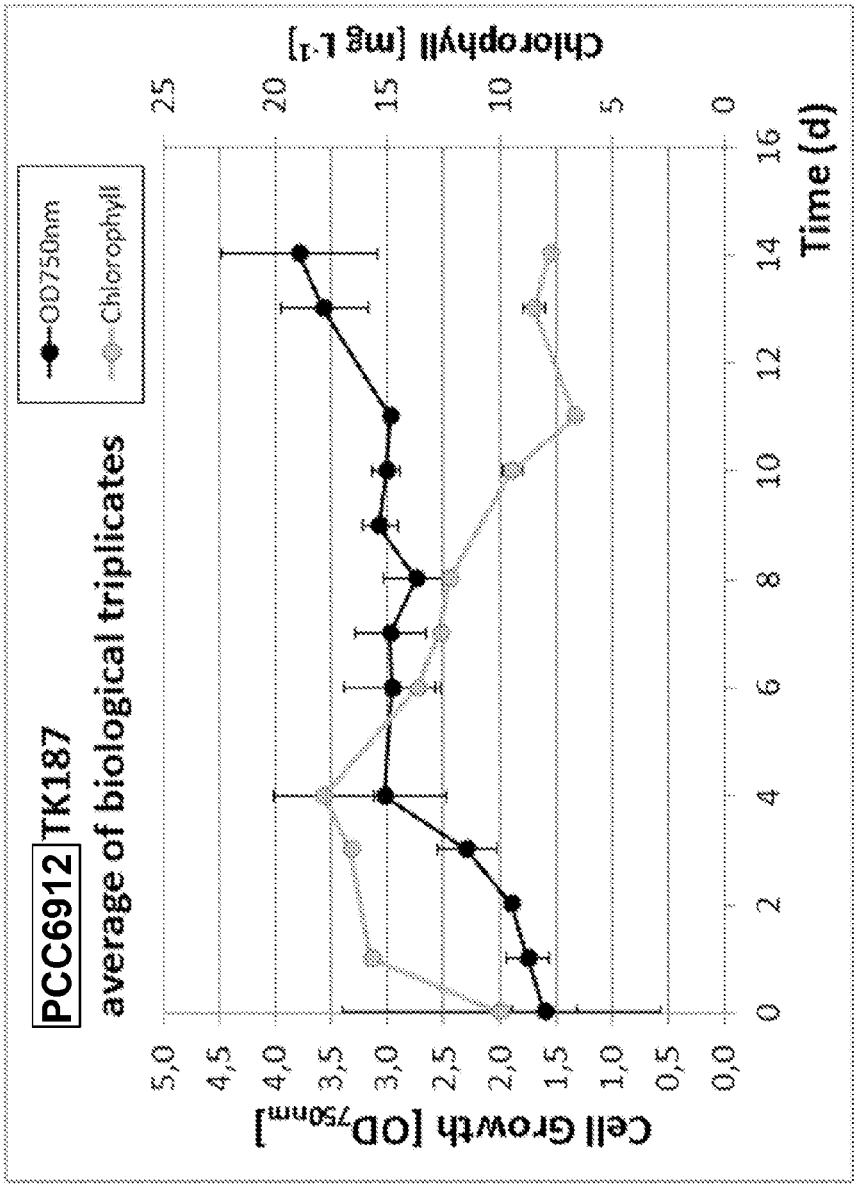

PCC6912 TK187
average of biological triplicates
OD750nm
Chlorophyll
Cell Growth [OD750nm]
Chlorophyll [mg L-1]
Time (d)
0,0
0,5
1,0
1,5
2,0
2,5
3,0
3,5
4,0
4,5
5,0
0
5
10
15
20
25
0
2
4
6
8
10
12
14
16

FIG. 7C

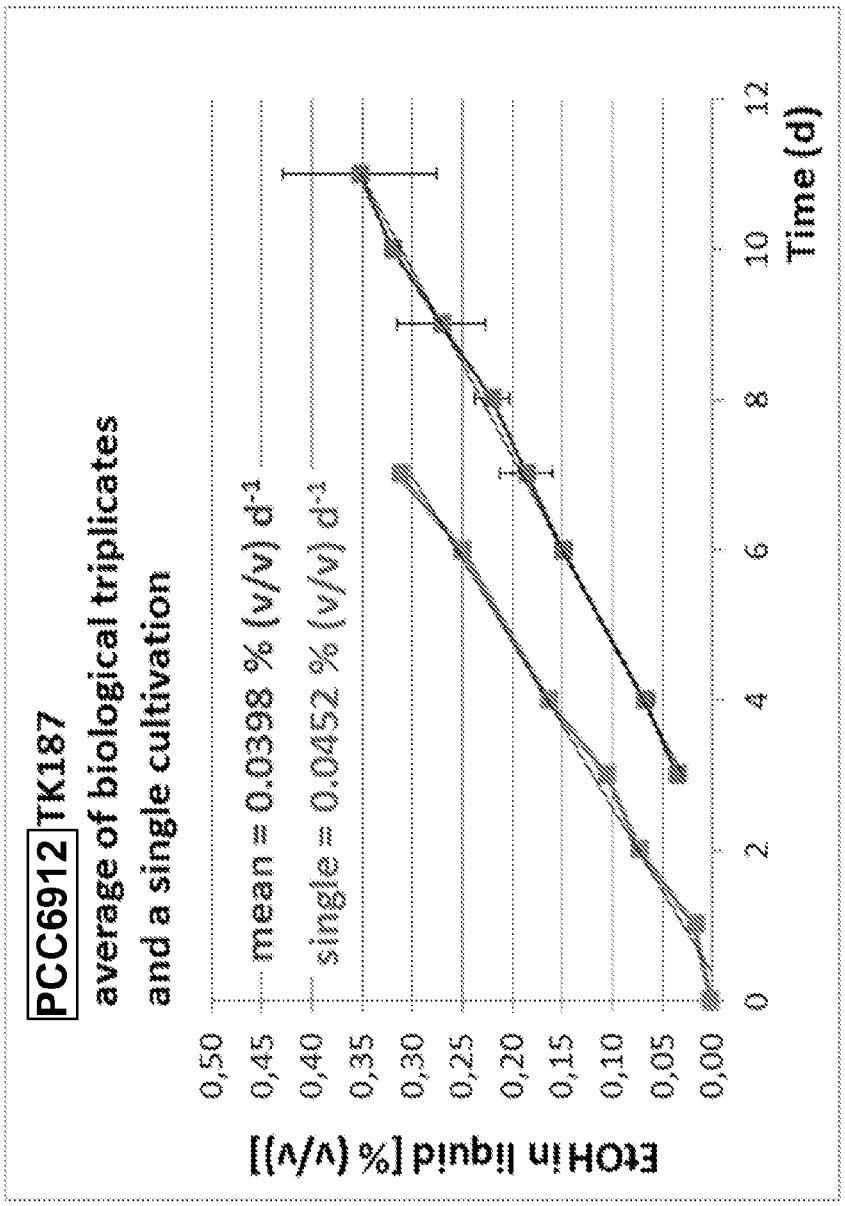
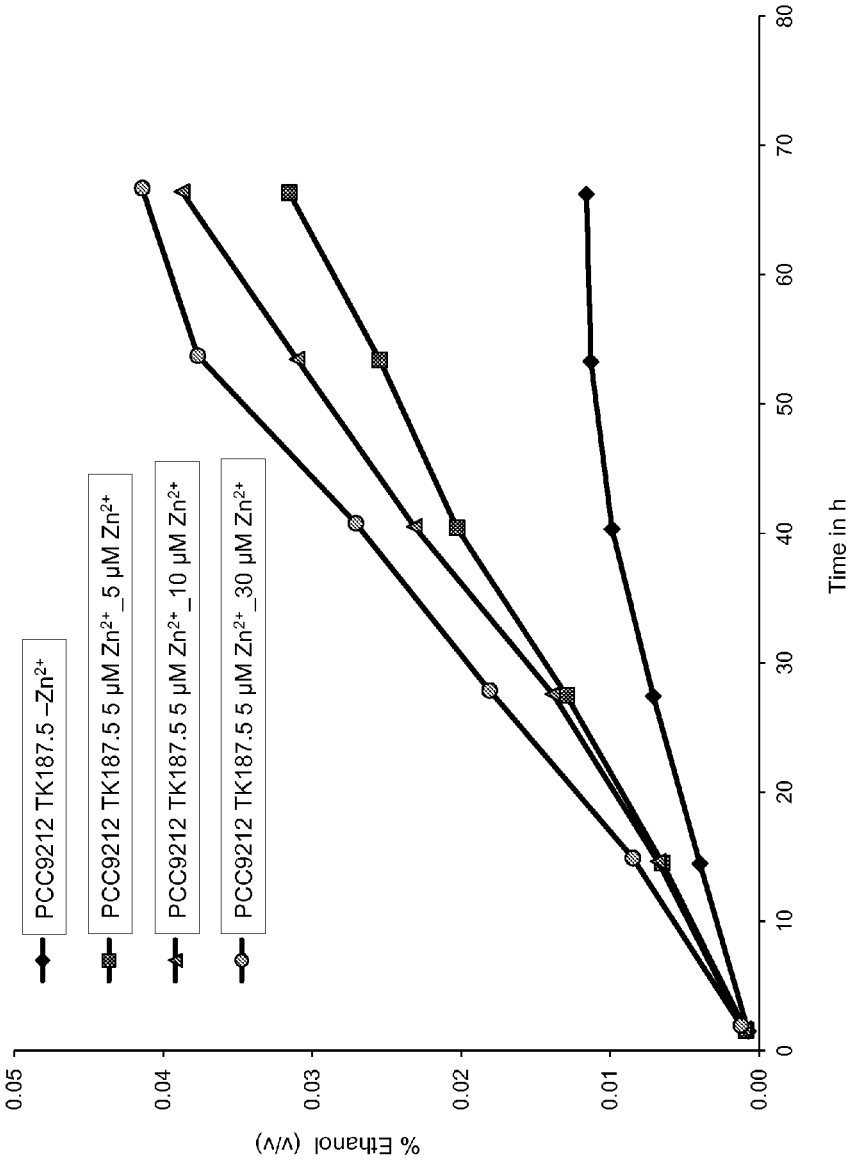

PCC6912 TK187
average of biological triplicates
and a single cultivation
mean = 0.0398 % (v/v) d-1
single = 0.0452 % (v/v) d-1
EtOH in liquid [% (v/v)]
0,50
0,45
0,40
0,35
0,30
0,25
0,20
0,15
0,10
0,05
0,00
0
2
4
6
8
10
12
Time (d)

FIG. 7D

FIG. 7E


Chlorogloeopsis PCC9212
PCC9212 TK187.5 –Zn2+
PCC9212 TK187.5 5 µM Zn2+_5 µM Zn2+
PCC9212 TK187.5 5 µM Zn2+_10 µM Zn2+
PCC9212 TK187.5 5 µM Zn2+_30 µM Zn2+
% Ethanol (v/v)
0.00
0.01
0.02
0.03
0.04
0.05
0
10
20
30
40
50
60
70
80
Time in h

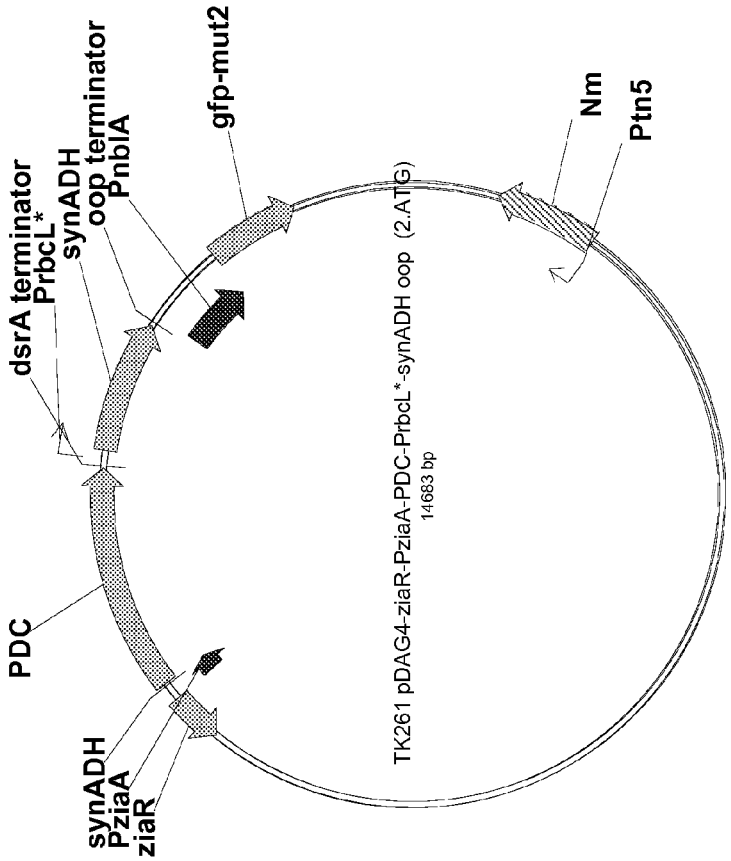

dsrA terminator
PrbcL*
synADH
oop terminator
PnblA
gfp-mut2
Nm
Ptn5
TK261 pDAG4-ziaR-PziaA-PDC-PrbcL*-synADH oop (2.ATG)
14683 bp
PDC
synADH
PziaA
ziaR

FIG. 8

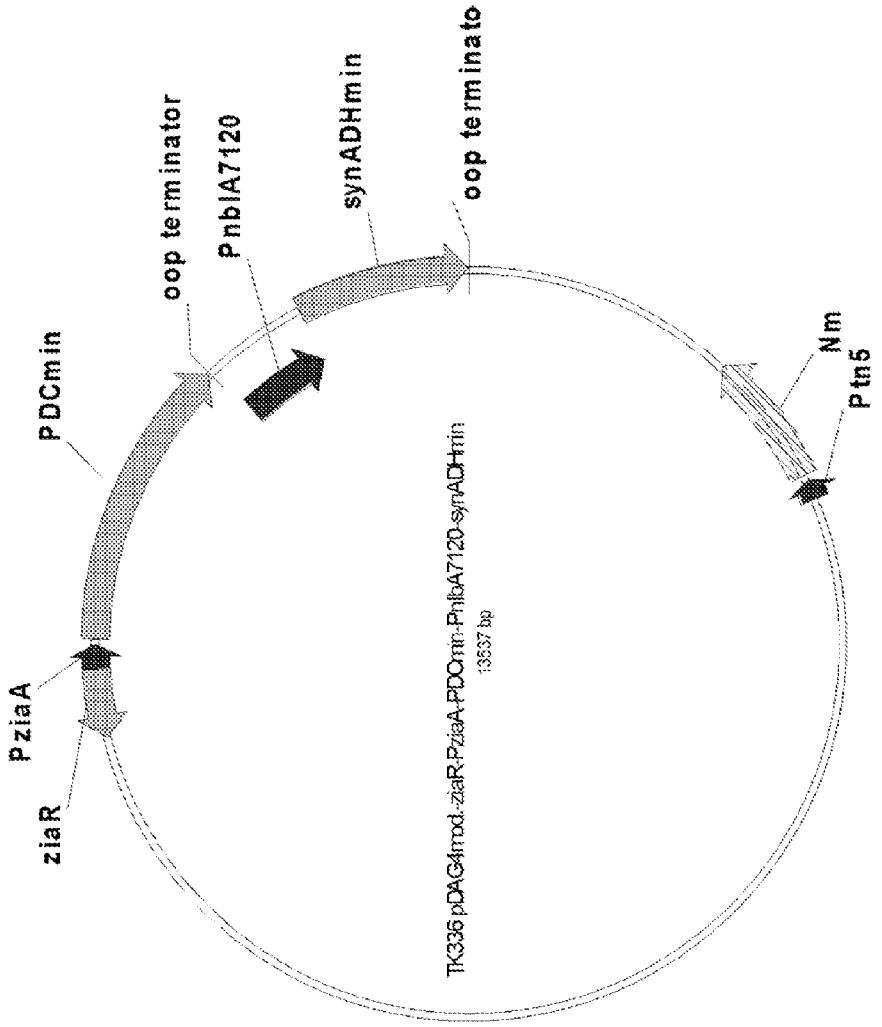

oop terminator
PnblA7120
synADHmin
oop terminato
PDCmin
Nm
Ptn5
PziaA
ziaR
TK336 pDAG4mod-ziaR-PziaA-PDCmin-PnlbA7120-synADHmin
13537 bp

FIG. 9A

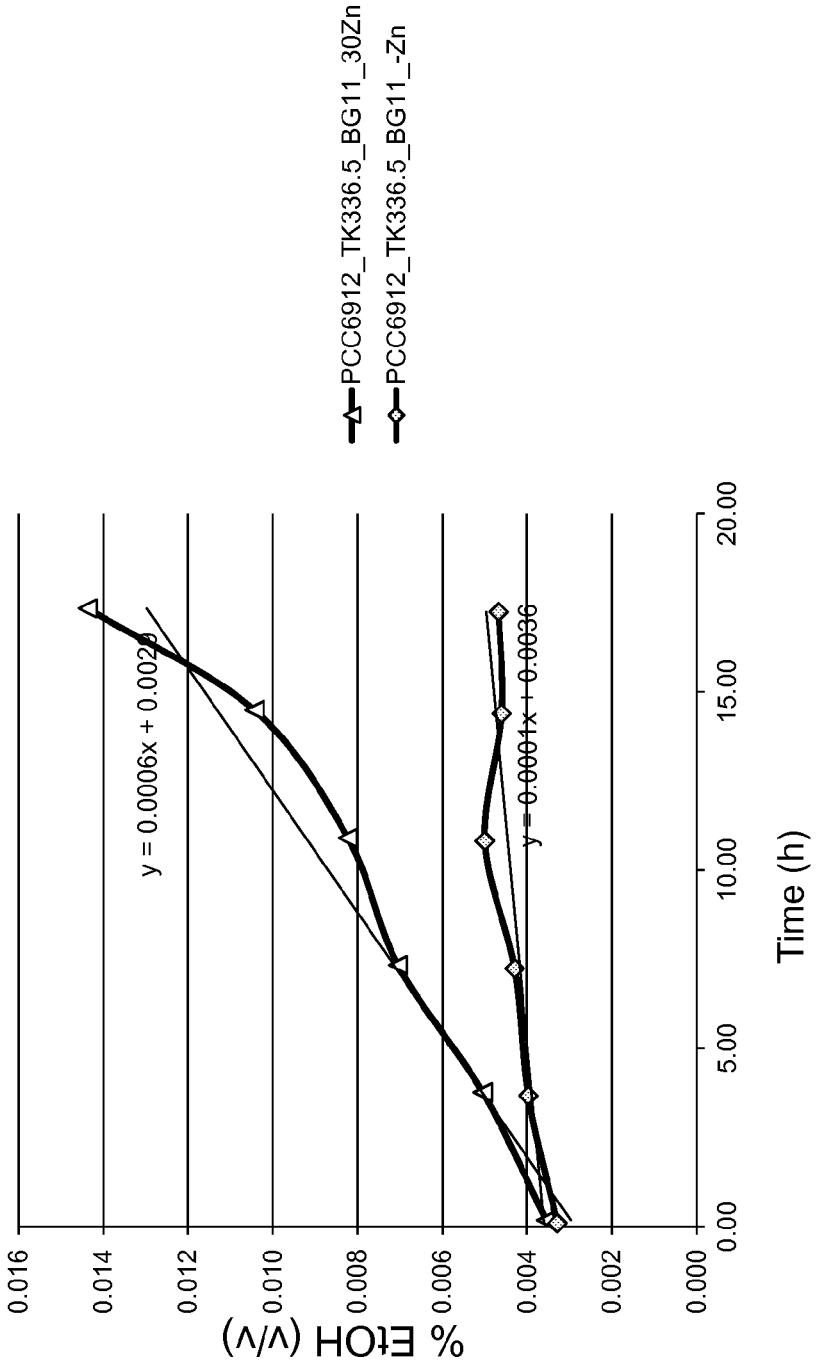

PCC6912_TK336.5_BG11_30Zn
PCC6912_TK336.5_BG11_-Zn
y = 0.0006x + 0.0022
y = 0.0001x + 0.0036
% EtOH (v/v)
Time (h)
0.016
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
0.00
5.00
10.00
15.00
20.00

FIG. 9B

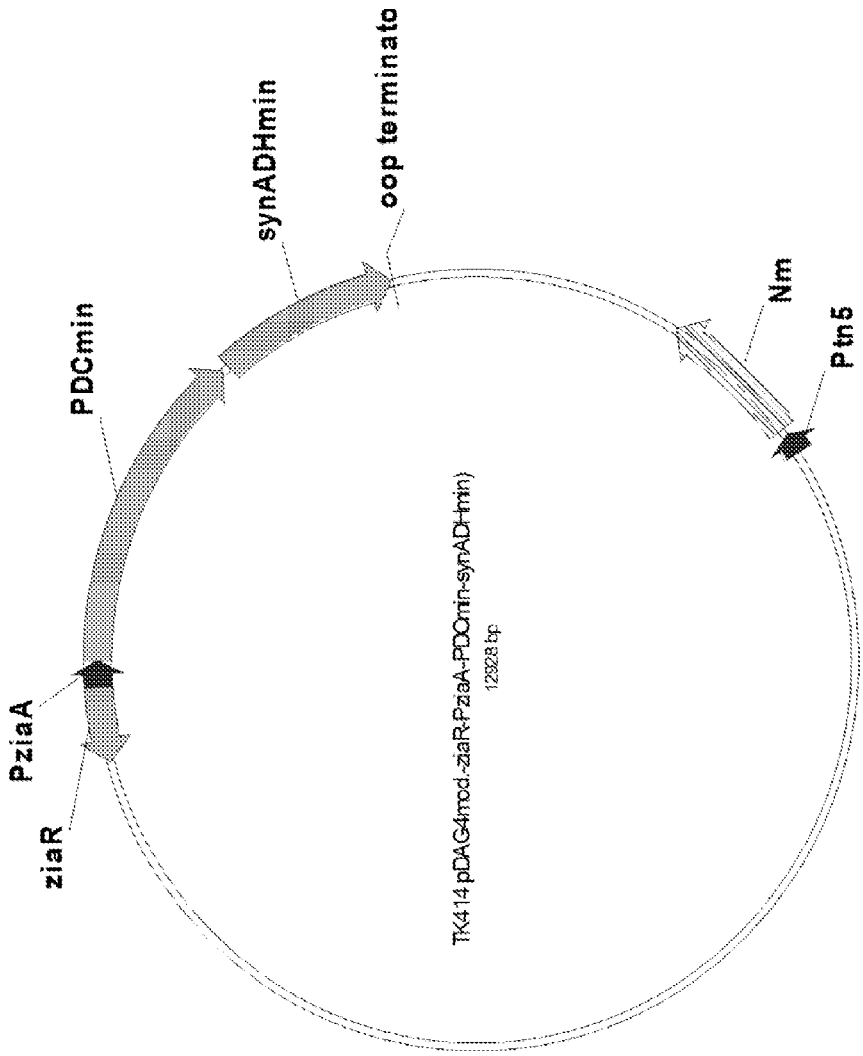

oop terminato
synADHmin
PDCmin
Nm
Ptn5
PziaA
ziaR
TK414 pDAG4mod.-ziaR-PziaA-PDCmin-synADHmin)
12928 bp

FIG. 10A

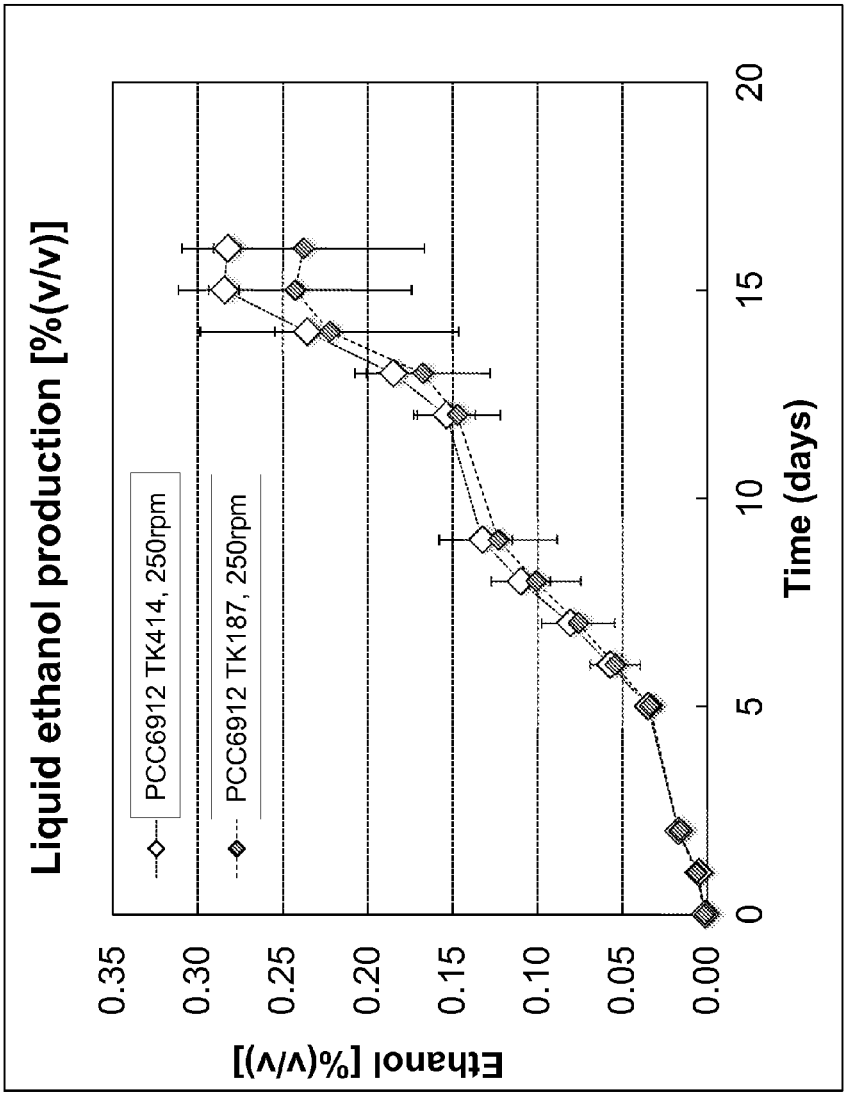
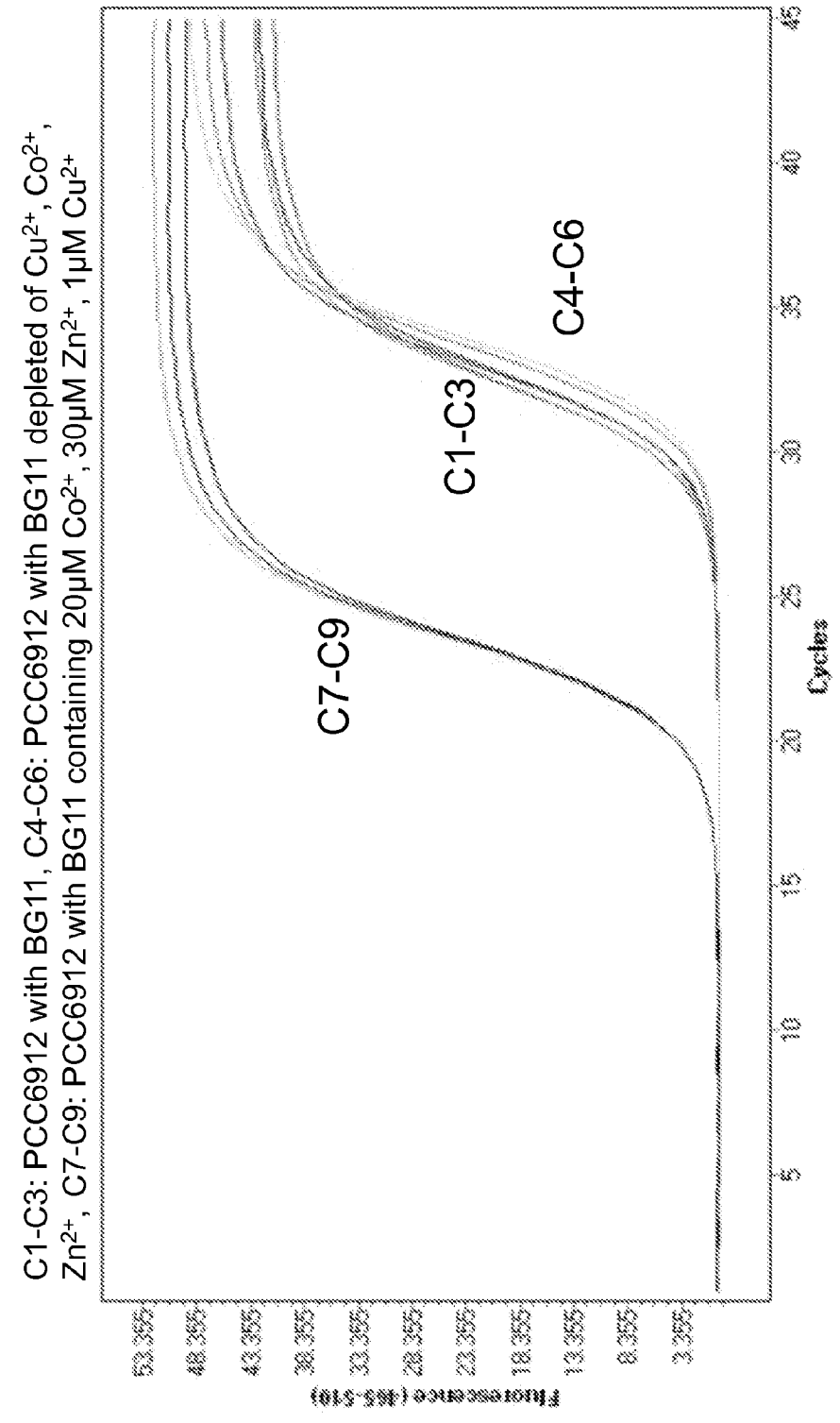

Liquid ethanol production [%(v/v)]
PCC6912 TK414, 250rpm
PCC6912 TK187, 250rpm
Ethanol [%(v/v)]
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.35
0
5
10
15
20
Time (days)

FIG. 10B

FIG. 11 A


Amplification curves
C1-C3: PCC6912 with BG11, C4-C6: PCC6912 with BG11 depleted of Cu^{2+}, Co^{2+}, Zn^{2+}, C7-C9: PCC6912 with BG11 containing 20μM Co^{2+}, 30μM Zn^{2+}, 1μM Cu^{2+}
C7-C9
C1-C3
C4-C6
Cycles
Fluorescence (465-510)

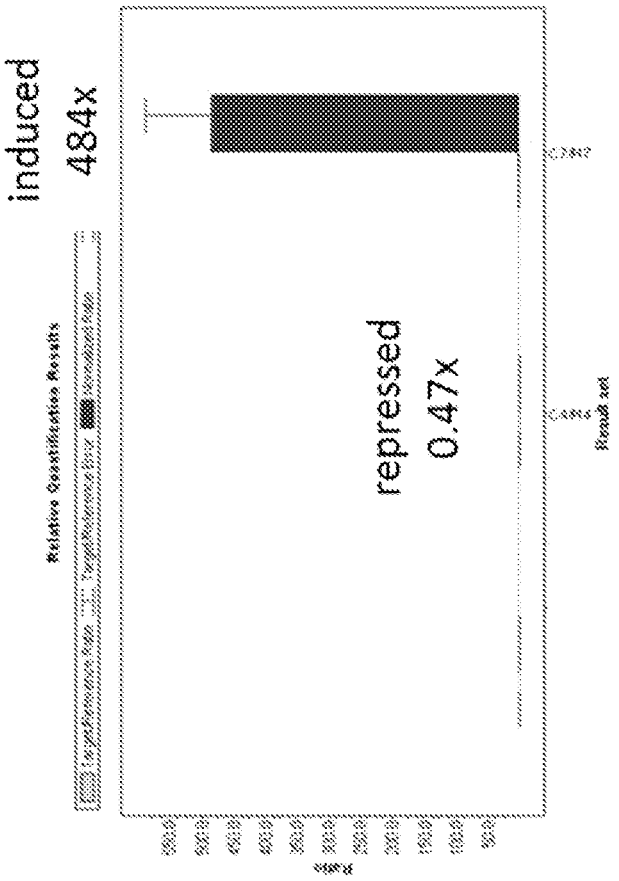


FIG. 11 B

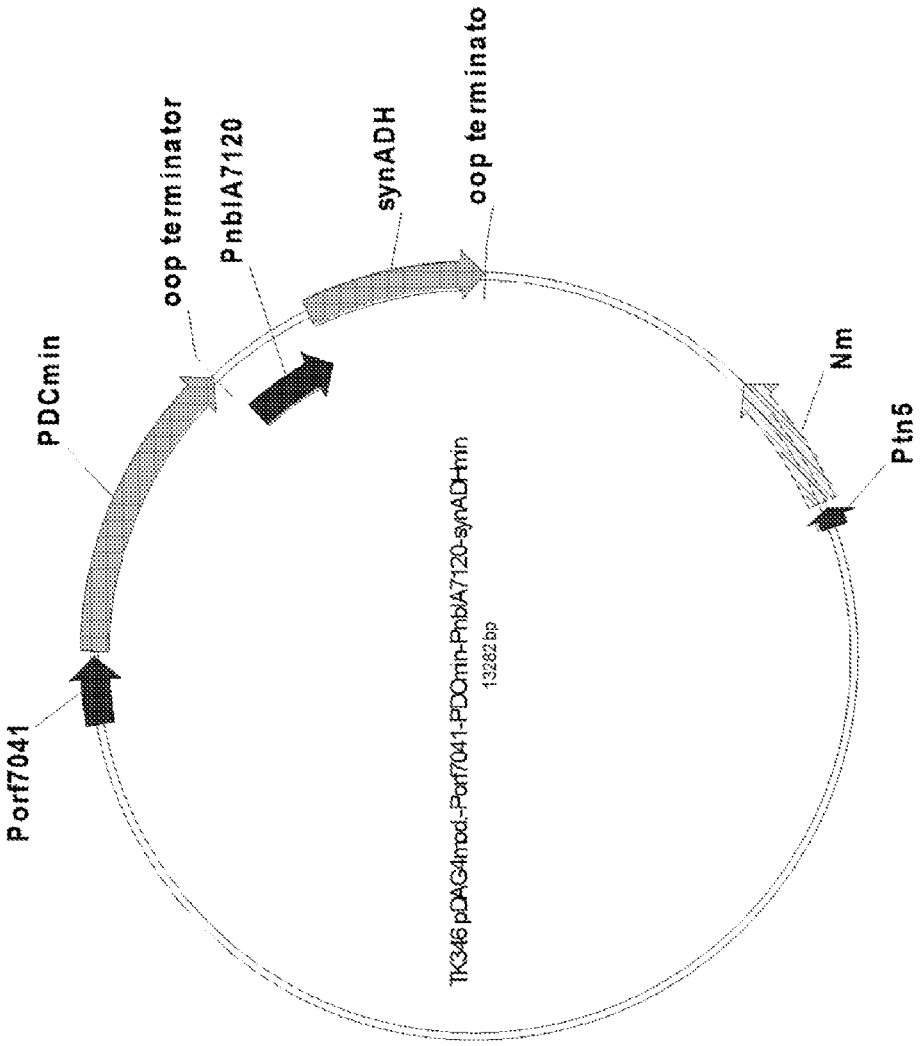

oop terminator
PnbiA7120
synADH
oop terminato
PDCmin
Nm
Ptn5
Porf7041
TK346 pDAG4mod.-Porf7041-PDCmin-PnbiA7120-synADH-tmin
13282 bp

FIG. 12A

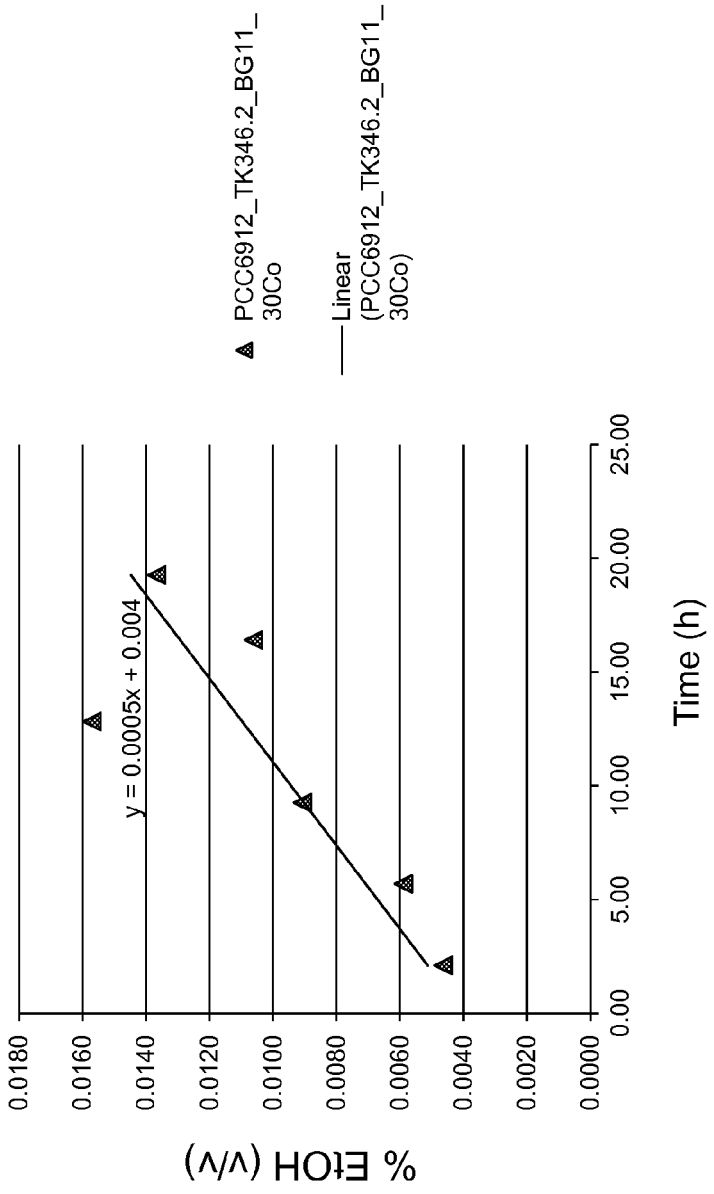

PCC6912_TK346.2_BG11_30Co
PCC6912_TK346.2_BG11_30Co
Linear (PCC6912_TK346.2_BG11_30Co)
y = 0.0005x + 0.004
% EtOH (v/v)
Time (h)
0.0180
0.0160
0.0140
0.0120
0.0100
0.0080
0.0060
0.0040
0.0020
0.0000
0.00
5.00
10.00
15.00
20.00
25.00

FIG. 12B

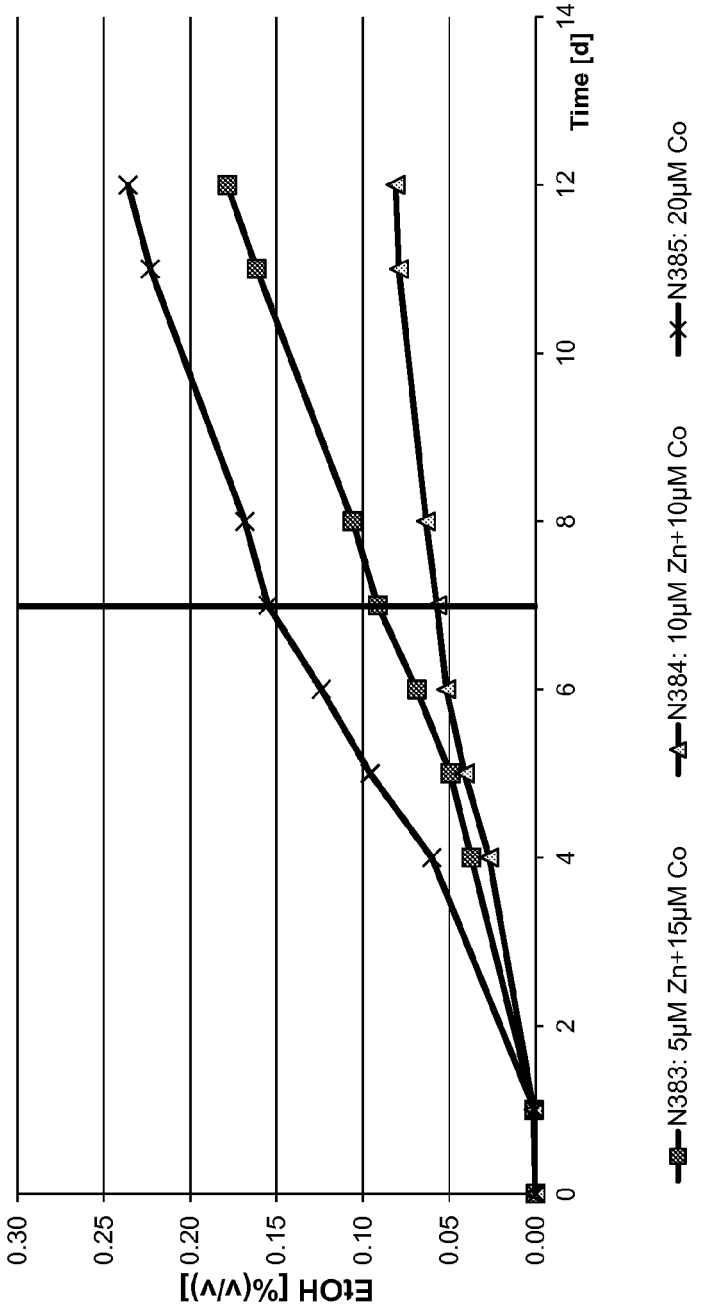

EtOH [%(v/v)]
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
2
4
6
8
10
12
14
Time [d]
N383: 5µM Zn+15µM Co
N384: 10µM Zn+10µM Co
N385: 20µM Co

FIG. 12C

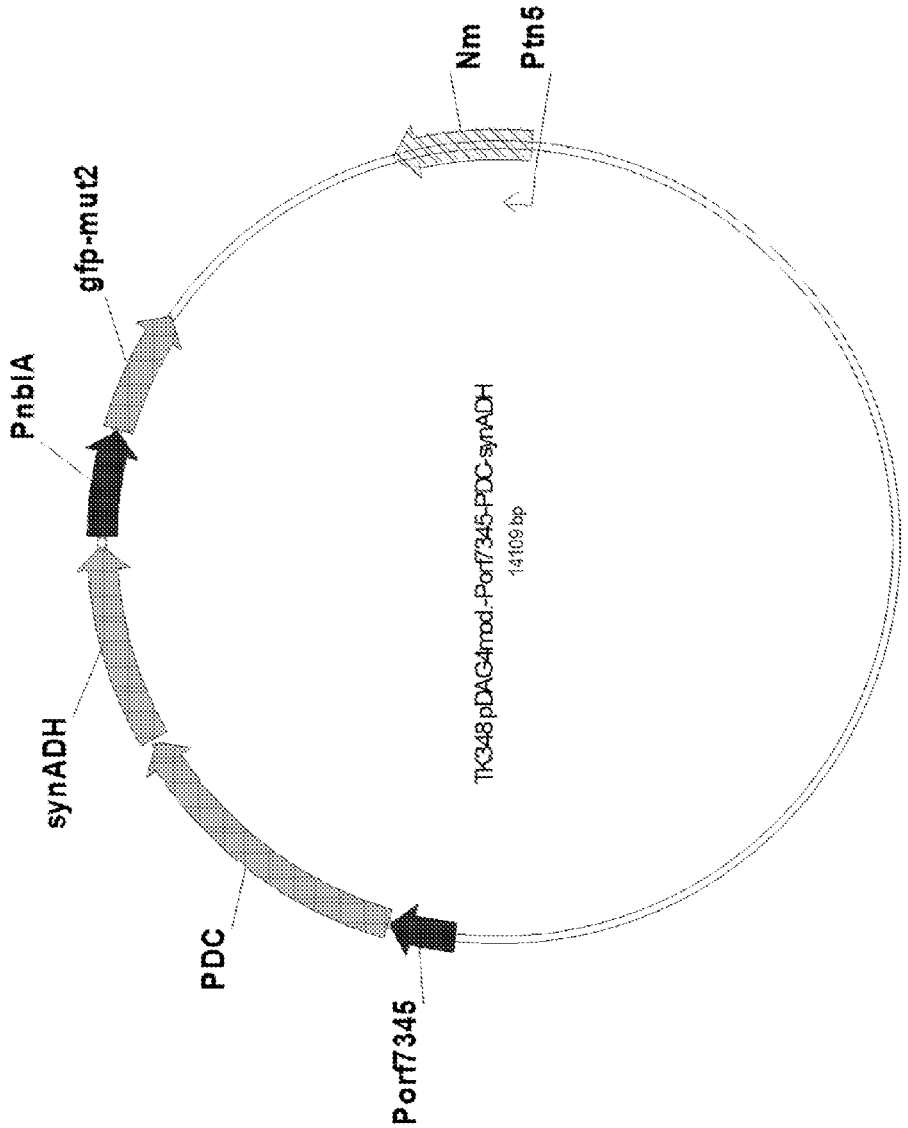

Nm
Ptn5
gfp-mut2
PnblA
synADH
PDC
Porf7345
TK348 pDAG4mod.-Porf7345-PDC-synADH
14169 bp

FIG. 13

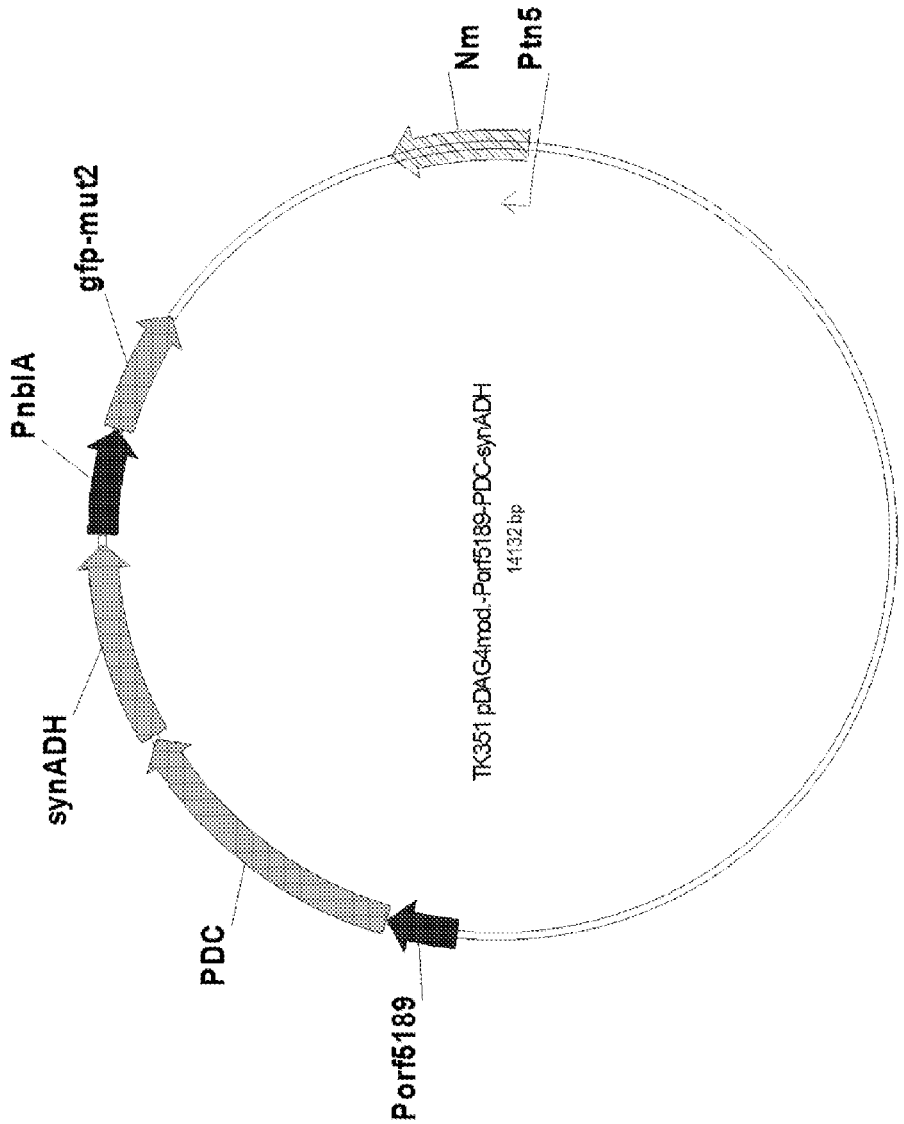

Nm
Ptn5
gfp-mut2
PnblA
synADH
PDC
Porf5189
TK351 pDAG4mod.-Porf5189-PDC-synADH
14132 bp

FIG. 14

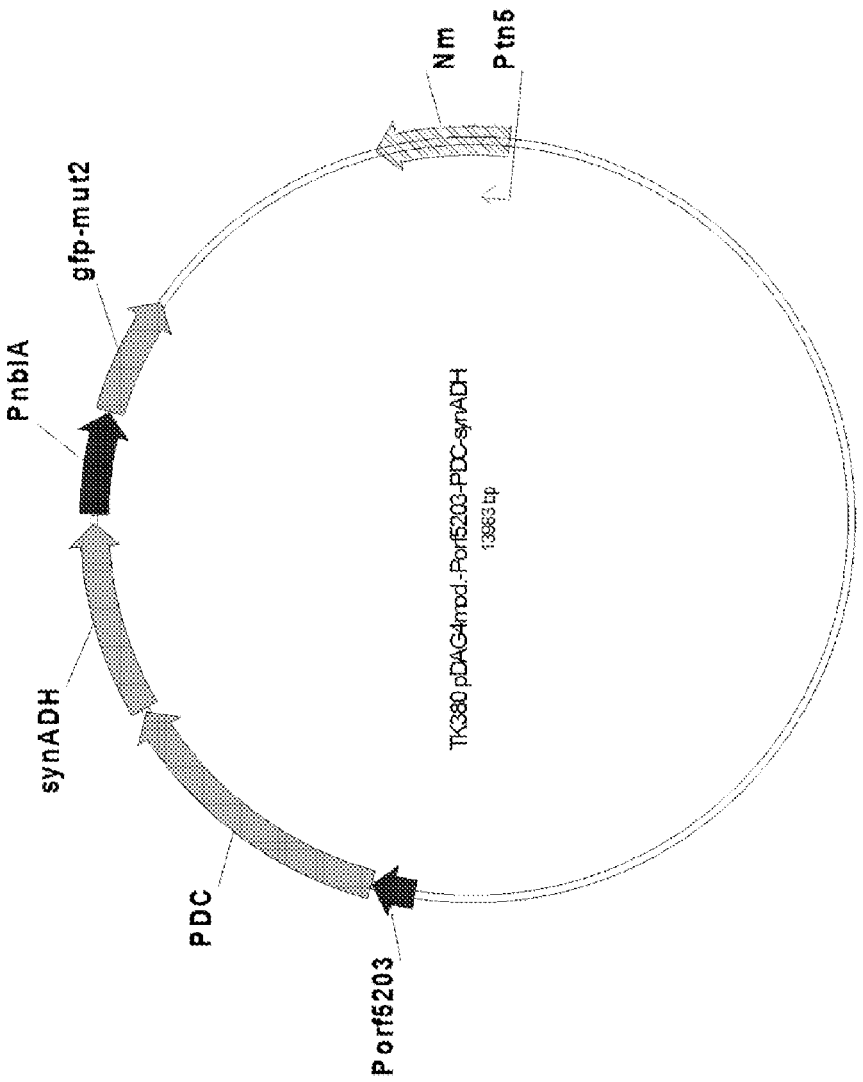

Nm
Ptn5
gfp-mut2
PnbiA
synADH
PDC
Porf5203
TK380 pDAG4mod.-Porf5203-PDC-synADH
13963 bp

FIG. 15

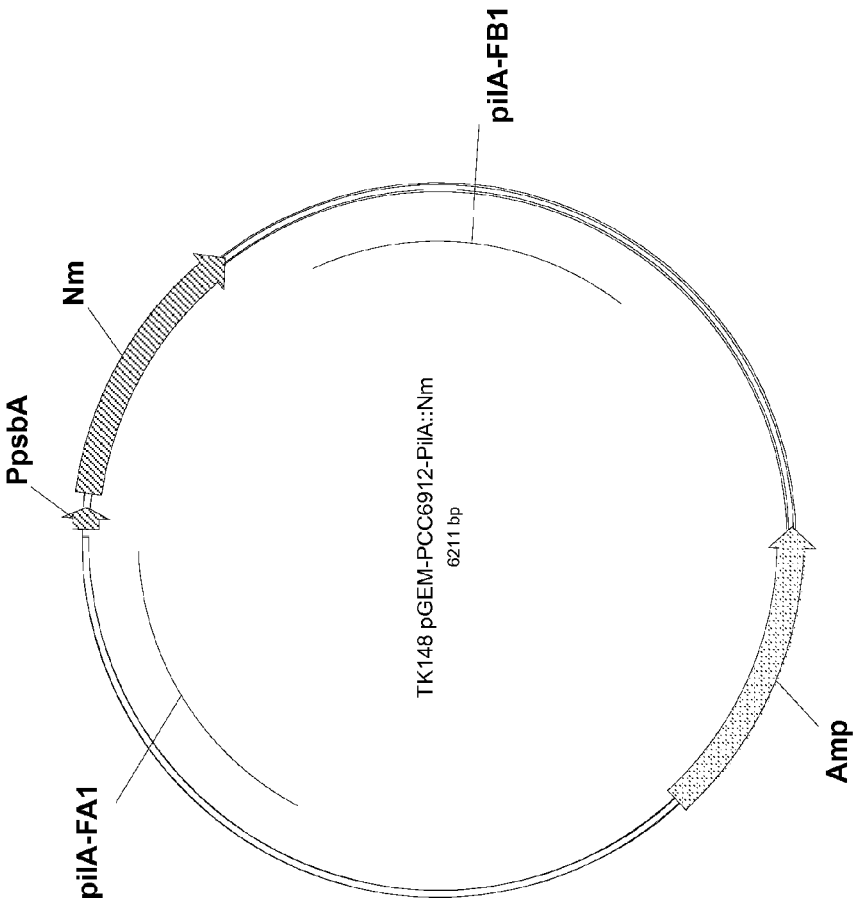

pilA-FB1
Nm
PpsbA
TK148 pGEM-PCC6912-PilA::Nm
6211 bp
pilA-FA1
Amp

FIG. 16A

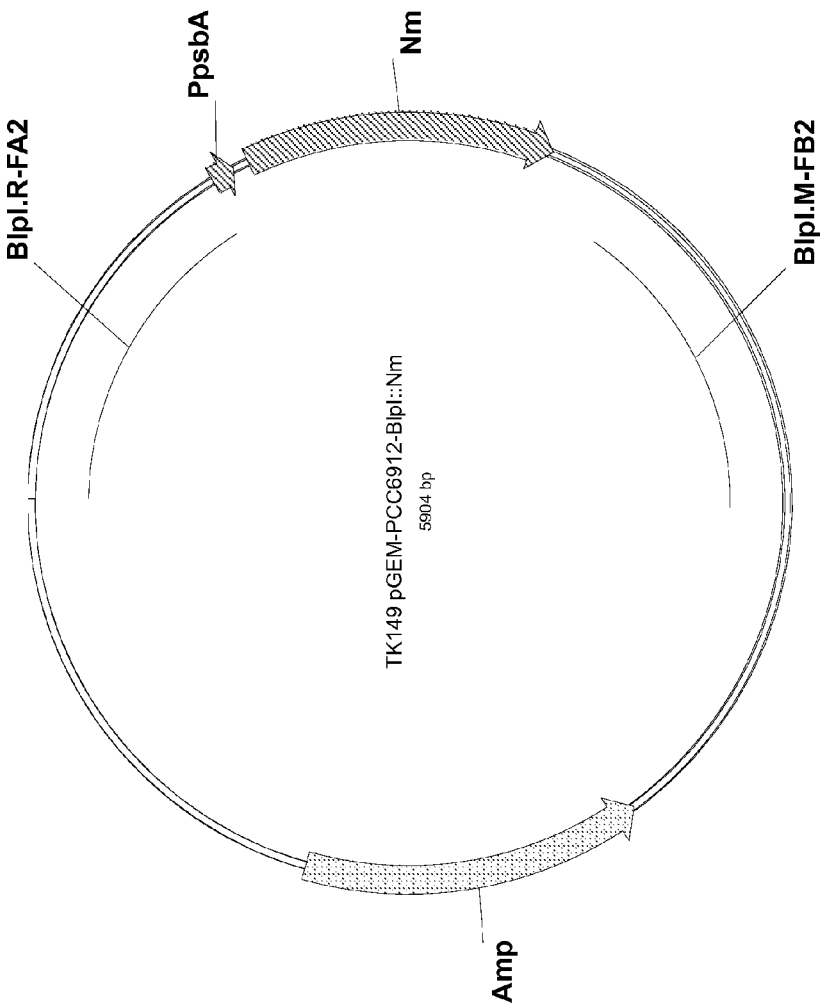

PpsbA
Nm
BlpI.R-FA2
BlpI.M-FB2
TK149 pGEM-PCC6912-BlpI::Nm
5904 bp
Amp

FIG. 16B

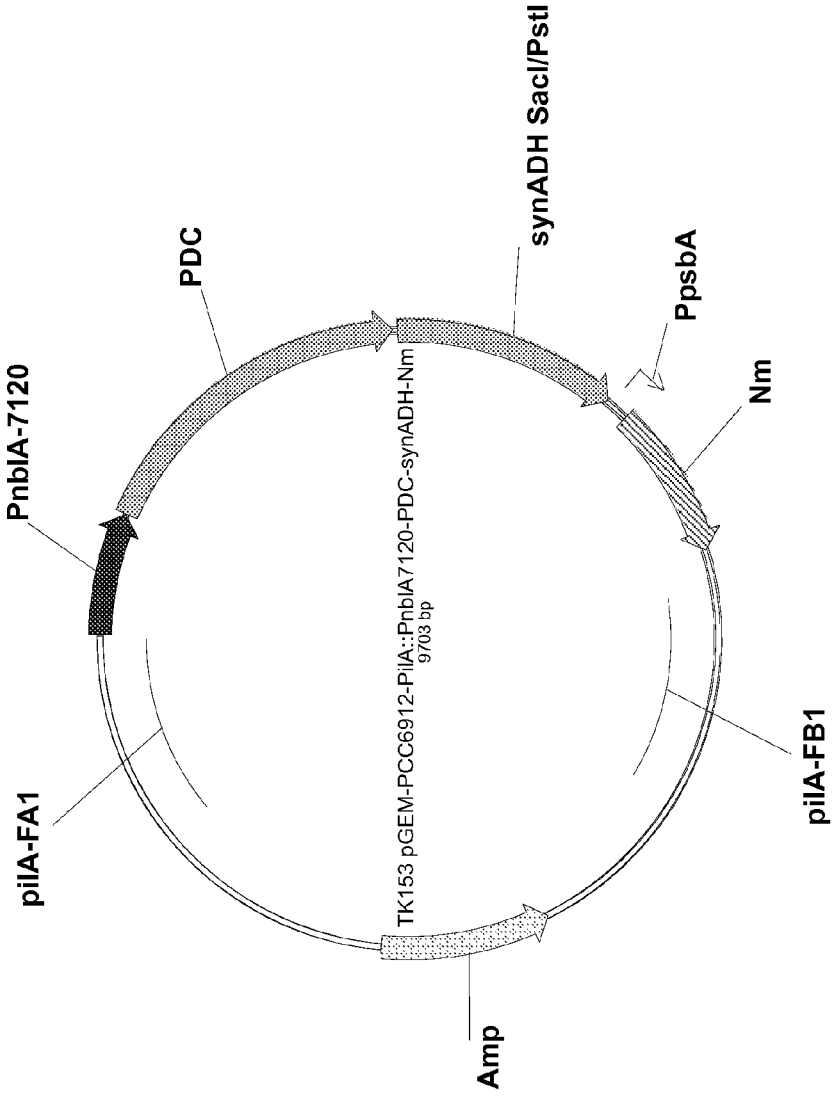

synADH SacI/PstI
PpsbA
Nm
pilA-FB1
Amp
pilA-FA1
PnblA-7120
PDC
TK153 pGEM-PCC6912-PilA::PnblA7120-PDC-synADH-Nm
9703 bp

FIG. 16C

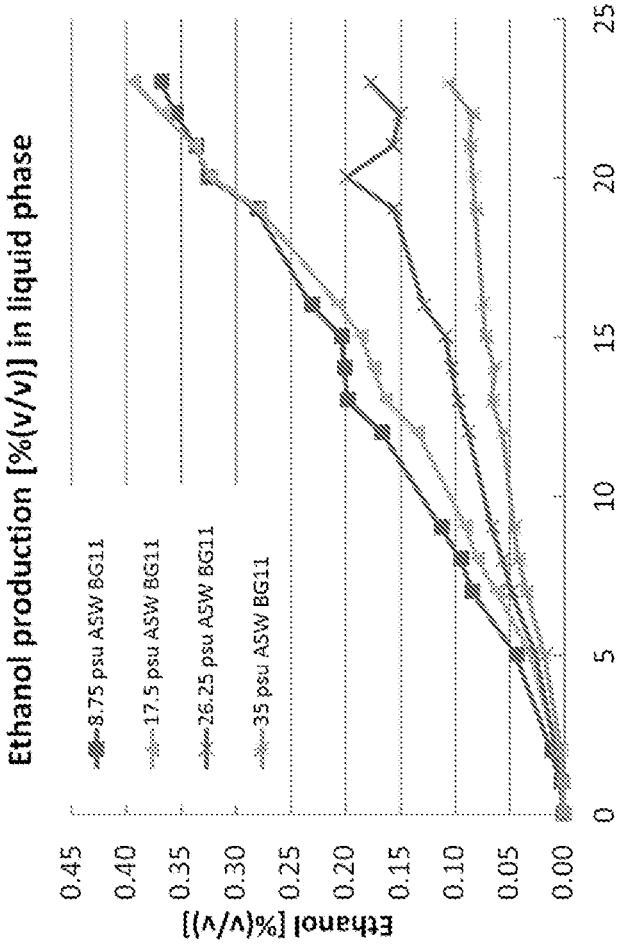

Ethanol production [%(v/v)] in liquid phase
8.75 psu ASW BG11
17.5 psu ASW BG11
26.25 psu ASW BG11
35 psu ASW BG11
Ethanol [%(v/v)]
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
5
10
15
20
25
Time (days)

FIG. 17A

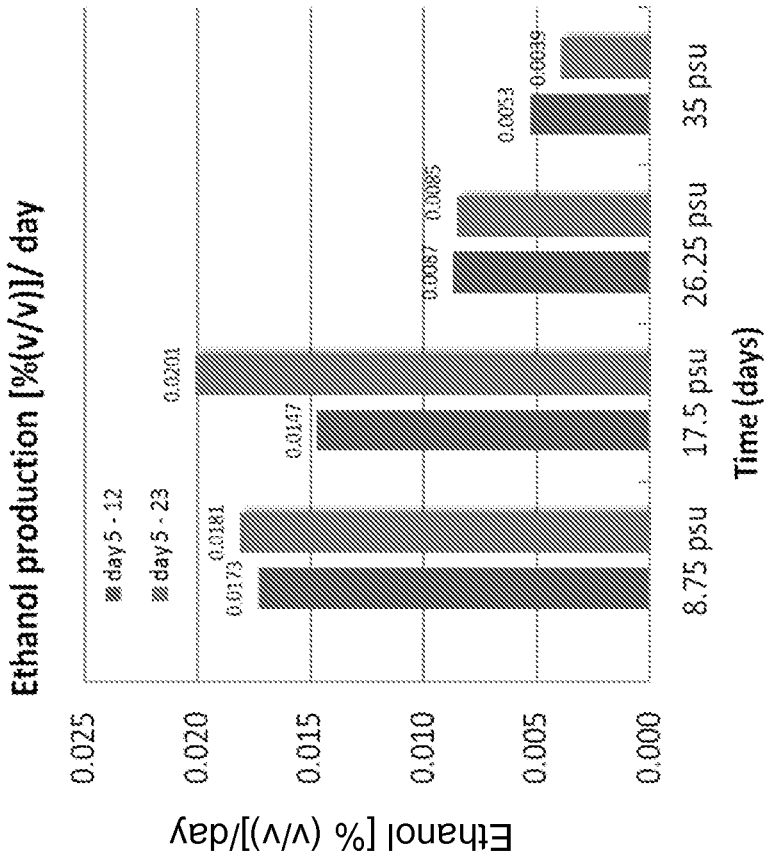

Ethanol production [%(v/v)]/ day
day5 - 12
day5 - 23
0.0173
0.0181
0.0147
0.0201
0.0087
0.0085
0.0053
0.0039
0.025
0.020
0.015
0.010
0.005
0.000
Ethanol [% (v/v)]/day
8.75 psu
17.5 psu
26.25 psu
35 psu
Time (days)

FIG. 17B

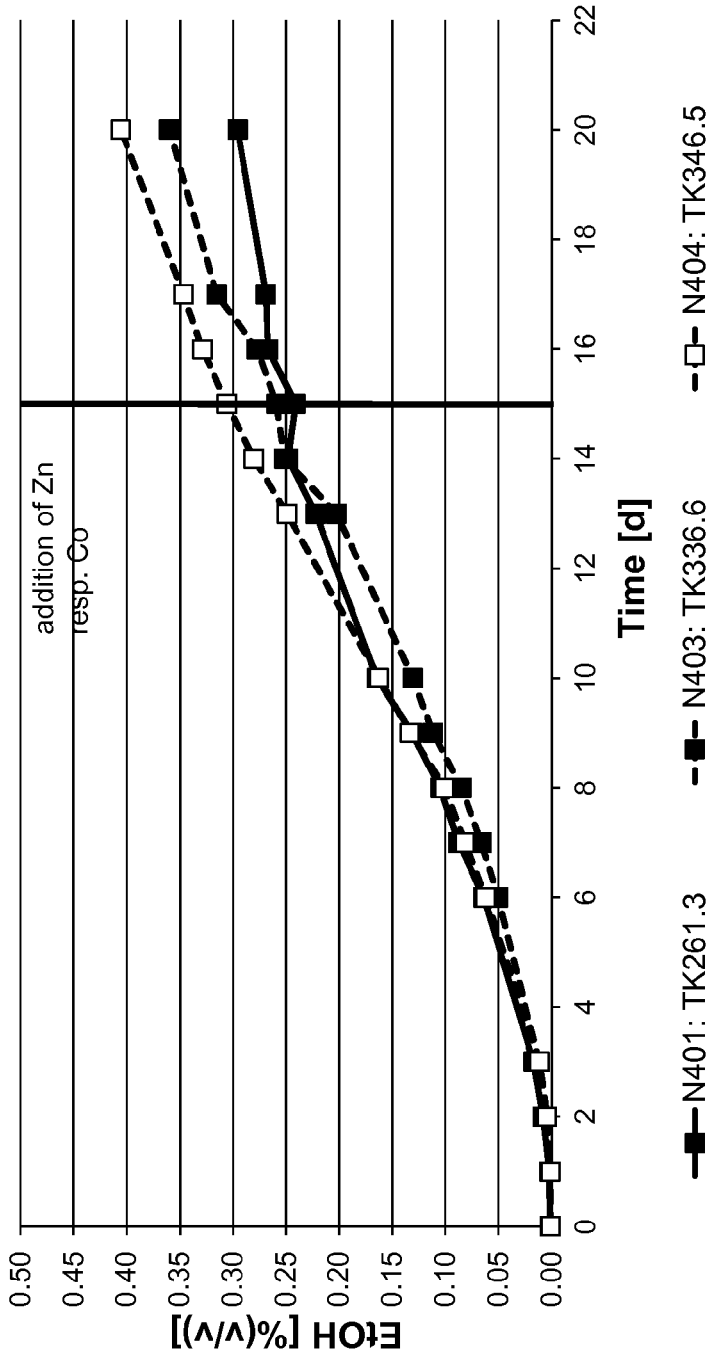

EtOH [%(v/v)]
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.35
0.40
0.45
0.50
Time [d]
0
2
4
6
8
10
12
14
16
18
20
22
addition of Zn
resp. Co
N401: TK261.3
N403: TK336.6
N404: TK346.5

FIG. 18

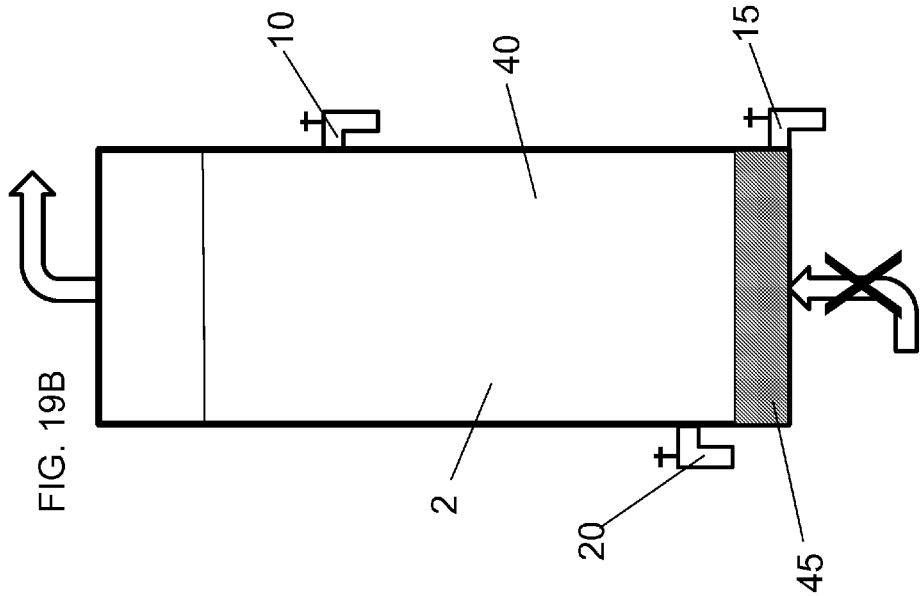


FIG. 19B

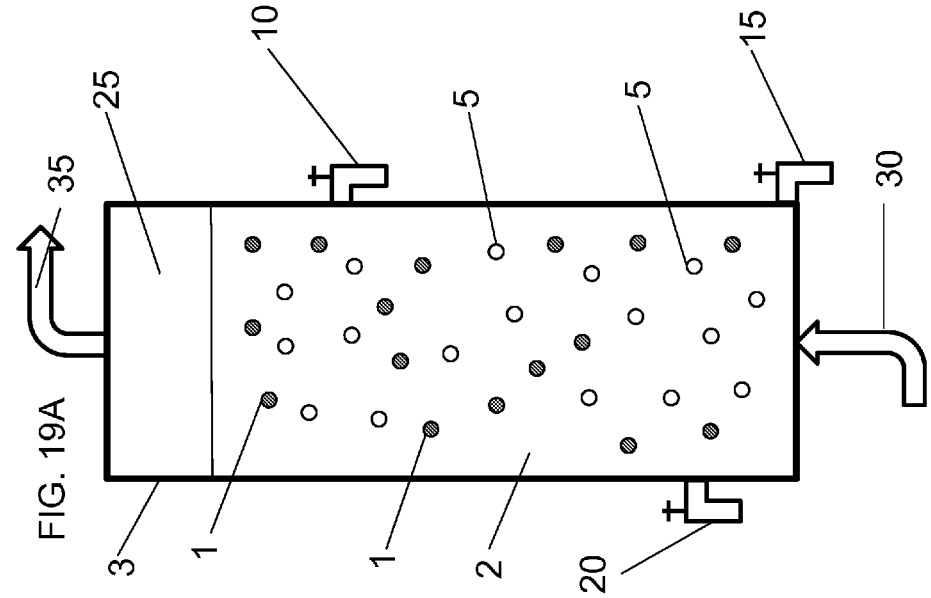


FIG. 19A

CHLOROGLOEOPSIS SP. HOST CELL FOR PRODUCING ETHANOL AND METHOD FOR PRODUCING ETHANOL USING THE SAME

CROSS-REFERENCE TO RELATED APPLICATIONS

This patent application is a continuation of International Application No. PCT/EP2013/077496, filed Dec. 19, 2013, the disclosure of which is hereby incorporated by reference.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

Not Applicable.

REFERENCE TO SEQUENCE LISTING

This application contains a sequence listing comprising 36 sequences, submitted by EFS-Web, thereby satisfying the requirements of 37 C.F.R. §§1.821-1.825. The sequence listing file, named "*Chlorogloeopsis*—_ST25.txt", was created on Aug. 25, 2015, and is 286 kb in size.

FIELD OF THE INVENTION

The present invention relates to the genetic enhancement of *Chlorogloeopsis* sp. host cells in order to produce ethanol as a compound of interest.

BACKGROUND OF THE INVENTION

Cyanobacteria are small, prokaryotic, generally aquatic organisms. Some cyanobacterial species can be genetically engineered in order to produce compounds of interest by utilizing light and carbon dioxide. These compounds of interest can include biofuels, industrial chemicals, pharmaceuticals, nutrients, carotenoids, food supplements and other compounds such as lipids. Owing to the fact that cyanobacteria are capable of fixing carbon dioxide as a carbon source for photoautotrophic growth, they do not require the input of organic carbon as feedstock and generally only need few nutrients. Some cyanobacterial species such as *Synechococcus* or *Synechocystis* have been genetically engineered in order to produce various compounds of interest such as ethanol (see for example U.S. Pat. No. 6,699,696 and U.S. Pat. No. 6,306,639, as well as PCT patent application WO 2009/098089 A2). Cyanobacterial cells can grow under a large variety of different growth conditions including sweet water as well as brackish water and can also thrive at very different temperatures.

The cyanobacterial genus *Chlorogloeopsis* belongs to the subsection V of cyanobacteria and is a heterocyst forming nitrogen fixing cyanobacterial genus, which can among others be isolated from hot springs (original publications: Mitra, A. A. and Pandey, D. C. (1967) "On a new genus of the blue-green alga *Chlorogloeopsis* with remarks on the production of heterocysts in the alga"; Phykos 5: pages 106 to 114 and Mitra, A. K. (1950): Two new algae from Indian soils. Ann. Bot. London. N. S. 14: 457-464).

The scientific publication Stucken et al.: "Transformation and Conjugal Transfer of Foreign Genes into the Filamentous Multicellular Cyanobacteria (Subsection V) *Fischerella* and *Chlorogloeopsis*"; Curr Microbiol., 2012 November; 65(5): 552-560, describes successful transformation of Cyanobacteria of subsection V by introducing the gene coding for the green fluorescent protein GFP into *Fischerella* and *Chlorogloeopsis* so that these cells were able to express the GFP reporter protein under two different promoters: the nitrogen regulated PglnA and the strong constitutive *E. coli* promoter Ptrc. For both strains partial removal of the exopolysaccharide sheath by salt washing was a critical step. However, the expression of the green fluorescent protein, which is not an enzyme, does not greatly affect the metabolism of the cyanobacterial cells because it does not consume metabolically important intermediates. This is in contrast to enzymes which are expressed to catalyze the production of chemical compounds of interest. Therefore this publication does not disclose any information on how a stable production of chemical compounds such as ethanol in *Chlorogloeopsis* can be achieved.

What is needed in the art is a new cyanobacterial strain for the production of ethanol, which can withstand hard culturing conditions and the metabolic stress associated with the production of chemical compounds of interest.

SUMMARY OF INVENTION

One aspect of the invention is directed to a genetically enhanced *Chlorogloeopsis* sp. host cell comprising at least one first recombinant gene encoding a first protein for the production of ethanol under the transcriptional control of a first inducible promoter, having at least 85%, 90% or 95% sequence identity to an endogenous inducible promoter of the *Chlorogloeopsis* sp. host cell.

Further, the genetically enhanced *Chlorogloeopsis* sp. host cell can be *Chlorogloeopsis fritschii* PCC6912, *Chlorogloeopsis* sp. PCC 9212, or *Chlorogloeopsis* sp. ABICyano3, preferably *Chlorogloeopsis fritschii* PCC6912.

A second aspect of the invention describes a method for producing ethanol, comprising the method steps of:

a) culturing the genetically enhanced *Chlorogloeopsis* sp. host cells described in the patent application in a culture medium, the host cells thereby producing ethanol,

b) retrieving ethanol at least from either one of: the host cells, the medium or the headspace above the medium.

In particular, the host cells are cultured under at least one of the following culturing conditions:

temperatures between 20° C. to about 55° C., preferably between 30° C. to 45° C., and/or

a salinity of the culture medium of between 0.2 to 35.0 psu, in particular 0.2, 5.0, 8.75 and 17.5 psu.

A third aspect of the invention is directed to a method for producing genetically enhanced *Chlorogloeopsis* sp. host cells comprising introducing a first and if present second recombinant gene into the host cell.

This method can comprise the method steps of:

a) providing a recombinant nucleic acid sequence including the first and if present second recombinant gene and protecting said recombinant nucleic acid sequence against endogenous restriction endonucleases of the host cell,

b) introducing the first and if present second recombinant gene into the host cell.

BRIEF DESCRIPTION OF THE DRAWINGS AND THE SEQUENCE LISTING

FIG. **1** shows a fluorescence photograph of the staining of *Chlorogloeopsis* PCC6912 cells with the lectin Concanavalin A-FITC (ConA-FITC) conjugated with a fluorescence marker showing the capsule or extracellular polymeric layer (EPS) of *Chlorogloeopsis* PCC6912. ConA-FITC can be used for labelling of carbohydrate moieties on the cell surface of the cyanobacterial cells.

FIG. **2**A and FIG. **2**B are agarose gels showing the presence of endogenous restriction endonucleases resulting in digestion of certain plasmids after incubation with the crude extracts of the *Chlorogloeopsis* sp. cells.

FIG. **3**A and FIG. **3**B show fluorescence photography of *Chlorogloeopsis* sp. ABICyano3 cells transformed with an extrachromosomal pDU1 based plasmid harboring the gene encoding the green fluorescent protein under the transcriptional control of a promoter inducible by nitrogen starvation. FIG. **3**A: non-induced; FIG. **3**B: induced by nitrogen-starvation.

FIG. **4**A shows the plasmid map of the pDU1 based plasmid TK18 harboring a gene encoding pyruvate decarboxylase enzyme and also a second recombinant gene encoding the alcohol dehydrogenase from *Synechocystis* PCC6803 under the transcriptional control of the petE promoter from *Nostoc* PCC7120, which was shown to be a constitutive promoter in *Chlorogloeopsis* PCC6912. This plasmid also includes PnblA controlling a gfp gene. The nucleotide sequence of this plasmid is shown in SEQ ID No. 1. The gene encoding the green fluorescent protein runs from nucleotides 628 to 1338, the complementary sequence of the gene conferring neomycin resistance "Nm" is located at nucleotides 2910 to 3701, the promoter PnblA stretches from the nucleotides 18 to 621, the gene encoding the *Synechocystis* alcohol dehydrogenase denoted "synADH" is located at the nucleotides 13201 to 14337, the gene encoding pyruvate decarboxylase denoted "PDC" runs from nucleotides 11472 to 13178, and the promoter PpetE from *Nostoc/Anabaena* PCC7120 labeled as "PpetE7120" runs from nucleotides 11124 to 11470.

FIG. **4**B shows the accumulation of ethanol measured via GC online experiments over a course of nearly 20 hours in *Chlorogloeopsis* PCC6912 cells harboring/containing the plasmid TK18.

FIG. **4**C depicts the accumulation of ethanol during the course of a 60 hour cultivation of *Chlorogloeopsis* PCC9212 including the plasmid TK18 measured via GC online experiments.

FIG. **5** shows the plasmid map of the plasmid TK122 including a Zn^{2+} inducible heterologous promoter PziaA from *Synechocystis* PCC6803 including its respective repressor ZiaR controlling the transcription of both a PDC and *Synechocystis* ADH enzyme encoding gene. This plasmid, similar to many other plasmids disclosed in this patent application, also includes a gene coding for a green fluorescent protein (gfp-mut2) under the control of the promoter PnblA from *Nostoc* 7120. In PCC6912 and PCC9212 the PnblA promoter is constitutive (which is not the case for *Chlorogloeopsis* sp. ABICyano3). Gfp was included to detect the presence of the plasmid in the cyanobacterial cells. The nucleotide sequence of the plasmid TK122 is shown in SEQ ID NO. 2. In this plasmid the following important genes are located: the gene coding for pyruvate decarboxylase "PDC" is located between nucleotides 5 to 1705, the *Synechocystis* alcohol dehydrogenase encoding gene "synADH" runs from nucleotides 1730 to 2867, and the promoter controlling the transcription of this gene PnblA is located at nucleotides 2900 to 3503, the promoter PziaA is between nucleotides 14132 to 14275 and the complementary sequence of the corresponding repressor gene ziaR runs from the nucleotides 13726 to 14124.

FIG. **6** shows two possible annotations for a start codon (ATG) of a protein encoding gene in a genomic region including a putative endogenous Zn^{2+} inducible promoter PziaA from *Chlorogloeopsis* PCC6912. The putative gene ziaA in *Chlorogloepsis* was identified based on the sequence homologies of the deduced protein to the Zn^{2+} transporting ATPase, ZiaA, from *Synechocystis* sp. PCC6803 (SEQ ID NO. 3). Cloning of the shorter region up to the first ATG, resulted in plasmid TK186 while cloning of the longer version up to the second ATG resulted in TK187. Hybrids harboring TK186 did not produce any ethanol whereas hybrids containing TK187 achieved high ethanol production rates.

FIG. **7**A shows the plasmid map of the plasmid TK187 including an endogenous Zn^{2+} inducible promoter from *Chlorogloeopsis* PCC6912, which controls in the PCC6912 genome a gene whose deduced protein shows homologies to ZiaA of PCC6803. The promoter was called/named in analogy to PCC6803 PziaA. In addition to PziaA from *Chlorogloeopsis* PCC6912, TK187 also includes the respective repressor gene ziaR. This promoter controls the transcription of both the first and second recombinant genes, encoding for PDC enzyme and *Synechocystis* alcohol dehydrogenase enzyme. The nucleotide sequence of the plasmid TK187 is shown in SEQ ID NO. 4. In this plasmid the following important genes are located: the gene coding for pyruvate decarboxylase "PDC" is located between nucleotides 11498 to 13198, the *Synechocystis* alcohol dehydrogenase encoding gene "synADH" runs from nucleotides 13223 to 14360, and the promoter controlling the transcription of this gene PnblA is located at nucleotides 33 to 636, the endogenous promoter PziaA is between nucleotides 11348 to 11492 and the complementary sequence of the corresponding endogenous repressor gene ziaR runs from the nucleotides 10945 to 11346.

FIG. **7**B to FIG. **7**D depict the accumulation of ethanol (FIG. **7**B and FIG. **7**D) (% (v/v), the activities of ADH enzyme and PDC enzyme (FIG. **7**B), the growth of the cells given by $OD_{750\ nm}$, (FIG. **7**C), and the chlorophyll content of cells of *Chlorogloeopsis* PCC6912 genetically enhanced with the plasmid TK187 in mBG11 medium (FIG. **7**C). FIG. **7**B shows the accumulation of ethanol over 14 days (day 1 to day 15). Data are given as an average of biological triplicates. The mean productivity of the three cultivations (0.0327% (v/v)/d) is also indicated. In addition the ethanol accumulation over the course of the first 7 days is shown (**7**D) for a single cultivation leading of a peak production rate of 0.0452% (v/v) d^{-1}. The highest ethanol production rate as an average of the three biological triplicates shown in FIG. **7**B, calculated from day 3 to 11 is also indicated (**7**D). *Chlorogloeopsis* PCC6912 cells genetically enhanced with the plasmid TK187 were cultivated in 0.5 L photobioreactors and ethanol concentration determined via GC single measurements.

FIG. **7**E shows the accumulation of ethanol measured via GC online measurements for *Chlorogloeopsis* PCC9212 cells also harboring the plasmid TK187 cultured for nearly 70 hours in the uninduced state (—Zn) and at different factors of induction via addition of different amounts of Zn^{2+} to the medium (5 μM Zn^{2+}, 10 μM Zn^{2+} and 30 μM Zn^{2+}).

FIG. **8** shows the plasmid map of the plasmid TK261 including the Zn^{2+} inducible promoter PziaA from *Chlorogloeopsis* PCC6912 only controlling the transcription of the PDC enzyme encoding first recombinant gene. The *Synechocystis* ADH enzyme encoding second recombinant gene is controlled by the constitutive promoter PrbcL. The nucleotide sequence of plasmid TK261 is shown in the sequence listing as SEQ ID NO. 5. Apart from the green fluorescent protein encoding gene and the neomycin resistance cassette, this plasmid includes from nucleotides 1418 to 2021 the promoter PnblA. Furthermore, the PDC gene runs from nucleotides 13152 to 178 and the promoter PziaA controlling this gene is located between nucleotides 12520 to 13153, and the respective complementary sequence of the repressor ziaR is located at nucleotides 12606 to 13007. The second recom-

binant gene for ethanol production encoding *Synechocystis* alcohol dehydrogenase is located between nucleotides 309 to 1319. Between both ethanologenic genes the terminator sequence "dsrA\terminator" is inserted between nucleotides 190 to 214 and the oop terminator between the *Synechocystis* ADH gene and the green fluorescent protein gene is at nucleotides 1349 to 1380.

FIG. **9**A shows the plasmid map of the plasmid TK336, which includes the Zn^{2+} inducible PziaA promoter and its respective repressor ziaR from *Chlorogloeopsis* PCC6912 controlling the transcription of a codon improved version of the first recombinant gene encoding PDC enzyme. Transcription of a codon improved version of the second recombinant gene coding for *Synechocystis* alcohol dehydrogenase enzyme is controlled by the constitutive promoter PnblA from *Nostoc* PCC7120. SEQ ID NO. 6 shows the nucleotide sequence of this plasmid. The codon improved version of the pdc gene runs from nucleotides 1 to 1702 and the promoter PziaA controlling this gene is located between nucleotides 13386 to 13529, and the respective complementary sequence of the repressor ziaR is located at nucleotides 12984 to 13385. The second recombinant codon improved gene for ethanol production encoding *Synechocystis* alcohol dehydrogenase is located between nucleotides 2344 to 3357. Between both ethanologenic genes the oop terminator is inserted between nucleotides 1708 to 1740.

FIG. **9**B is a graph showing the ethanol accumulation in cultures of *Chlorogloeopsis* PCC6912 harboring the plasmid TK336 over a time course of around 18 hours in the uninduced (0Zn) and the induced state (30 μM Zn^{2+}) measured via GC online measurements.

FIG. **10**A shows the plasmid map of the plasmid TK414 including the Zn^{2+} inducible promoter PziaA from *Chlorogloeopsis* PCC6912 (nucleotides 12777 to 12920) controlling the transcription of codon improved variants of pdc (nucleotides 12921 to 1702) and adh genes (nucleotides 12921 to 1702). In addition a terminator sequence (oop terminator between nucleotides 2754 to 2786) is located downstream of the *Synechocystis* ADH enzyme encoding gene in order to ensure a reliable transcription termination. SEQ ID NO. 7 shows the nucleotide sequence of this plasmid.

FIG. **10**B includes a graph depicting a comparison of the ethanol accumulation (% (v/v) of *Chlorogloeopsis* PCC6912 hybrids containing the different plasmids TK414 and TK187 during 15 day cultivation in 0.5 liter photobioreactor. Ethanol concentration was determined via GC single measurements.

FIG. **11**A shows the metal-ion dependent induction of orf7041 by qRT-PCR. qRT-PCR shows the significant upregulation of orf7041 by addition of the metal-ion mix containing 20 μM Co^{2+}, 30 μM Zn^{2+}, and 1 μM Cu^{2+}. The promoter of orf7041 can be considered as being regulated by at least one of these metal-ions.

FIG. **11**B shows the relative quantification of orf7041 based on the amplification curves of the qRT-PCR. Expression levels were normalized to expression of a reference gene.

FIG. **12**A depicts the plasmid map of the plasmid TK346 including the promoter of the open reading frame (orf) 7041 from *Chlorogloeopsis* PCC6912 running from nucleotides 12898 to 13274, which is both a Co^{2+}/Zn^{2+} inducible promoter, but which mainly reacts to Co^{2+} controlling the transcription of a codon improved version of the first recombinant gene encoding PDC enzyme (from nucleotides 1 to 1702). The *Synechocystis* ADH enzyme encoding second recombinant gene (from nucleotides 2344 to 3357) is controlled by the constitutive promoter PnblA from *Nostoc* (nucleotides 1747 to 2343) and a transcription terminator sequence (oop terminator between nucleotides 1708 to 1740) is present between both recombinant genes in order to decouple the transcriptional control of these genes. The DNA sequence of this plasmid is shown in the sequence listing as SEQ ID NO. 8.

FIG. **12**B is a graph showing the ethanol accumulation over a 20 hour cultivation of an induced culture of *Chlorogloeopsis* PCC6912 (30 μM Co^{2+}) harboring the plasmid TK346 determined via GC online measurements.

FIG. **12**C depicts the ethanol accumulation over the time course of 12 days of the same hybrid as shown in FIG. **12**B in larger 0.5 liter photobioreactors determined via GC single measurements. The best productivity was observed with 20 μM Co^{2+}.

FIG. **13** shows the plasmid map of the plasmid TK348, including the promoter controlling the open reading frame (orf) 7345 of *Chlorogloeopsis* PCC6912 (from nucleotides 10832 to 11212), which is a promoter inducible by Zn^{2+} and Co^{2+}, but mainly reacts to Zn^{2+} and which controls the transcription of both the pdc (nucleotides 11220 to 12920) and the adh gene (nucleotides 12945 to 14082) encoding first and second recombinant enzymes. The DNA sequence of this plasmid is shown in the sequence listing as SEQ ID NO. 9.

FIG. **14** shows the plasmid map of the plasmid TK351 including the Zn^{2+} inducible promoter of the open reading frame (orf) 5189 of *Chlorogloeopsis* PCC6912 (running from nt 10832 to 11237) controlling the transcription of both the PDC enzyme (nucleotides 11243 to 12943) and *Synechocystis* ADH enzyme (nucleotides 12968 to 14105) encoding genes. SEQ ID NO. 10 depicts the nucleotide sequence of this plasmid.

FIG. **15** shows the plasmid map of the plasmid TK380 including the Zn^{2+} inducible promoter of the open reading frame (orf) 5203 of *Chlorogloeopsis* PCC6912 (running from nucleotides 10832 to 11088), controlling the transcription of both the first and second recombinant gene encoding PDC (nucleotides 11094 to 12794) and ADH enzyme (nucleotides 12819 to 13956). The nucleotide sequence of this plasmid is shown as SEQ ID NO. 11 in the sequence listing.

The plasmid map plasmid TK148 is shown in FIG. **16**A. This plasmid cannot replicate in *Chlorogloeopsis*. It contains a neomycin resistance conferring gene (denoted Nm running from nucleotides 121 to 902) under the transcriptional control of PpsbA (nucleotides 21 to 81), which is flanked by two sequences which are homologous to parts of the chromosomal pilA gene of *Chlorogloeopsis fritschii* PCC6912. The pilA parts are needed for homologous recombination of the neomycin conferring resistance gene into the genome of the *Chlorogloeopsis* sp. host cells (platform pilA-FB1 from nucleotides 1115 to 2196 and platform pilA-FA1 from nucleotides 5140 to 6210). SEQ ID NO. 12 shows the DNA sequence of this plasmid.

FIG. **16**B depicts the plasmid map of the plasmid TK149, which, similar to plasmid TK148, harbors a neomycin resistance conferring gene (from nucleotides 1053 to 1834) under the transcriptional control of PpsbA (nucleotides 953 to 1013). This resistance gene is flanked by two sequences homologous to parts of the gene BlpI. The BlpI part is necessary for integration of the neomycin resistance conferring gene into the genome of the *Chlorogloeopsis* sp. host cells (platform BlpI.M FB2 from nucleotides 2047 to 2962 and platform—BlpI.R FA2\ from nt 2 to 931). SEQ ID NO. 13 shows the DNA sequence of this plasmid.

The plasmid map of the plasmid TK153 is shown in FIG. **16**C. This plasmid is similar to TK148, but contains in addition an ethanologenic gene cassette including genes coding for PDC enzyme (nucleotides 626 to 2330) and ADH enzyme (nucleotides 2355 to 3492) under the transcriptional control of the promoter PnblA from *Nostoc/Anabaena* PCC7120

(nucleotides 6 to 622). Similar to plasmid TK148, two sequences for homologous recombination into the gene pilA are present upstream and downstream of the ethanologenic cassette and the neomycin resistance gene (denoted pilA-FA1) running from nucleotides 8632 to 9702 and pilA-FB1 (running from nucleotides 4607 to 5688). The nucleotide sequence of this plasmid is shown in the sequence listing as SEQ ID NO. 14.

FIG. **17**A shows the ethanol accumulation over a course of 23 days in *Chlorogloeopsis* PCC6912 hybrids containing the plasmid TK336 in medium at different salinities of 8.75, 17.5, 26.25 and 35 psu. Artificial seawater BG11 medium was used and modified to different salinities (aswBG11).

FIG. **17**B shows a comparison of the ethanol production rate (% (v/v)d^{-1}) between days 5 to 12 and days 5 to 23 for the same experiment already shown in FIG. **17**A at different salinities measured via GC single measurements.

FIG. **18** shows the ethanol accumulation in 35 psu medium (artificial mBG11) at pH 8 of parallel cultivations of *Chlorogloeopsis* PCC6912 hybrids harboring the plasmids TK261, TK336 and TK346, respectively.

FIG. **19**A is a schematic diagram showing a culture of PCC6912 and ABICyano3 during mixing of the culture.

FIG. **19**B is a schematic diagram showing the settling of a culture of PCC6912 and ABICyano3 to the bottom of the container after mixing of the culture medium is discontinued.

DETAILED DESCRIPTION OF INVENTION

Several strains of the genus *Chlorogloeopsis* sp. were successfully transformed for the first time with plasmids by using conjugation and electroporation procedures, resulting in ethanol production. This task was achieved by a new transformation protocol taking into consideration the specifics of the genus *Chlorogloeopsis* sp., such as protection of the plasmids used for transformation against the endogenous restriction endonucleases SphI and BlpI. Surprisingly, no special treatment such as sonification or salt washing steps for transformation via conjugation was necessary for the EPS layer around the *Chlorogloeopsis* sp. cells in order for the plasmids to be introduced into the cyanobacteria. In particular, the individual species *Chlorogloeopsis* PCC6912 and *Chlorogloeopsis* PCC9212 as well as *Chlorogloeopsis* ABICyano3 could be genetically enhanced with ethanologenic plasmids. Although these cyanobacterial strains belong to the same genus *Chlorogloeopsis* sp., they show differences in their cultivation behavior as well as in their sensitivity to salinity and other typical growth parameters.

Chlorogloeopsis PCC6912 and *Chlorogloeopsis* ABICyano3 were shown to form aggregates during cultivation, whereas *Chlorogloeopsis* PCC9212 was more uniformly dispersed in the culture medium. *Chlorogloeopsis fritschii* PCC6912 was able to produce ethanol at reasonable quantities in a wide temperature range of between 20° C. to about 55° C. and/or at salinities of the culture medium of between 0.2 to 35.0 psu, in particular 0.2, 8.75 and 17.5 psu, which was not the case for the other two cyanobacterial strains, which required freshwater medium for ethanol production. In contrast to *Chlorogloeopsis* sp. PCC9212, ABICyano3 showed a higher salt tolerance, which was however not comparable to *Chlorogloeopsis fritschii* PCC6912. This makes *Chlorogloeopsis* PCC6912 especially suitable for cultivation in deserts where a large temperature difference between day and night is to be expected. Furthermore *Chlorogloeopsis* PCC6912 showed high ethanol production rates at medium salinities between 0.2, 8.75 and 17.5 psu, so that this strain can also be cultivated in a brackish medium.

GENERAL EXPLANATIONS AND DEFINITIONS

Aspects of the invention utilize techniques and methods common to the fields of molecular biology, microbiology and cell culture. Useful laboratory references for these types of methodologies are readily available to those skilled in the art. See, for example, Molecular Cloning: A Laboratory Manual (Third Edition), Sambrook, J., et al. (2001) Cold Spring Harbor Laboratory Press; Current Protocols in Microbiology (2007) Edited by Coico, R, et al., John Wiley and Sons, Inc.; The Molecular Biology of Cyanobacteria (1994) Donald Bryant (Ed.), Springer Netherlands; Handbook Of Microalgal Culture Biotechnology And Applied Phycology (2003) Richmond, A.; (ed.), Blackwell Publishing; and "The cyanobacteria, molecular Biology, Genomics and Evolution", Edited by Antonia Herrero and Enrique Flores, Caister Academic Press, Norfolk, UK, 2008.

It is well known to a person of ordinary skill in the art that large plasmids can be produced using techniques such as the ones described in the U.S. Pat. No. 6,472,184 B1 titled "method for producing nucleic acid polymers" and U.S. Pat. No. 5,750,380 titled "DNA polymerase mediated synthesis of double stranded nucleic acid molecules", which are hereby incorporated in their entirety.

Denominations of genes are in the following presented in a three letter lower case name followed by a capitalized letter if more than one related gene exists, for example ziaA. The respective protein encoded by that gene is denominated by the same name with the first letter capitalized, such as ZiaA.

Denominations for promoter sequences, which control the transcription of a certain gene in their natural environment are given by a capitalized letter "P" followed by the gene name according to the above described nomenclature, for example "PnblA" for the promoter controlling the transcription of the nblA gene.

Denominations for enzyme names can be given in a two or three letter code indicating the origin of the enzyme, followed by the above mentioned three letter code for the enzyme itself, such as SynADH (Zn^{2+} dependent Alcohol dehydrogenase from *Synechocystis* PCC6803), ZmPdc (pyruvate decarboxylase from *Zymomonas mobilis*).

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art to which this invention belongs. As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

The term "about" is used herein to mean approximately, in the region of, roughly, or around. When the term "about" is used in conjunction with a numerical value/range, it modifies that value/range by extending the boundaries above and below the numerical value(s) set forth. In general, the term "about" is used herein to modify a numerical value(s) above and below the stated value(s) by a variance of 20%, 10% or 5%.

The term "Cyanobacteria" refers to a member from the group of photoautotrophic prokaryotic microorganisms which can utilize solar energy and fix carbon dioxide. Cyanobacteria are also referred to as blue-green algae.

The term "terminator" refers to a nucleic acid sequence which is able to terminate the transcription of an mRNA. The terminators can exert their function in various ways including, but not limited to forming a hairpin structure in the mRNA transcript, which disrupts the mRNA-DNA RNA polymerase complex during transcription or via forming a recognition site for a transcription termination factor. Non-limiting examples are dsrA from *E. coli*, the oop terminator or the rho terminator.

The term "*Chlorogloeopsis* sp." refers to an unspecified cyanobacterial member of the genus *Chlorogloeopsis*, which was among other characterized by Mitra, A. A. and Pandey, D. C. (1967) "On a new genus of the blue-green alga *Chlorogloeopsis* with remarks on the production of heterocysts in the alga"; Phykos 5: pages 106 to 114 and Mitra, A. K. (1950): Two new algae from Indian soils. Ann. Bot. London. N. S. 14: 457-464.

The terms "host cell" and "recombinant host cell" are intended to include a cell suitable for metabolic manipulation, e.g., which can incorporate recombinant polynucleotide sequences, e.g., which can be transformed. The term is intended to include progeny of the cell originally transformed. In particular embodiments, the cell is a prokaryotic cell, e.g., a cyanobacterial cell. The term recombinant host cell is intended to include a cell that has already been selected or engineered to have certain desirable properties and suitable for further enhancement using the compositions and methods of the invention.

The term "genome" refers to the chromosomal genome as well as to extrachromosomal plasmids which are normally present in the wild type *cyanobacterium* without having performed recombinant DNA technology. For example, cyanobacteria such as *Synechococcus* PCC7002 can include at least up to 6 extrachromosomal plasmids in their wild type form.

"Competent to express" refers to a host cell that provides a sufficient cellular environment for expression of endogenous and/or exogenous polynucleotides.

As used herein, the term "genetically enhanced" refers to any change in the endogenous genome of a wild type cell or to the addition of non-endogenous genetic code to a wild type cell, e.g., the introduction of a heterologous gene. More specifically, such changes are made by the hand of man through the use of recombinant DNA technology or mutagenesis. The changes can involve protein coding sequences or non-protein coding sequences, including regulatory sequences such as promoters or enhancers.

As used herein, the term "recombinant" refers to nucleic acid sequences and in particular to genes which are changed by laboratory methods thereby creating combinations of nucleic acid sequences in a host cell which are not found in the respective wild type host cell. This term can apply nucleic acid sequences which are both endogenous as well as heterologous with respect to the host cell.

The nucleic acids of this present invention may be modified chemically or biochemically or may contain non-natural or derivatized nucleotide bases, as will be readily appreciated by those of skill in the art. Such modifications include, for example, labels, methylation, substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as uncharged linkages, charged linkages, alkylators, intercalators, pendent moieties, modified linkages, and chelators. Also included are synthetic molecules that mimic polynucleotides in their ability to bind to a designated sequence via hydrogen bonding and other chemical interactions.

The term "homology" refers to the percentage of identity between two polynucleotide or two polypeptide moieties. The correspondence between the sequences from one moiety to another can be determined by techniques known to the art. For example, homology can be determined by a direct comparison of the sequence information between two polypeptide molecules by aligning the sequence information and using readily available computer programs. Alternatively, homology can be determined by hybridization of polynucleotides under conditions that form stable duplexes between homologous regions, followed by digestion with single-stranded-specific nuclease(s) and size determination of the digested fragments.

As used herein, "substantially similar" refers to nucleic acid fragments wherein changes in one or more nucleotide bases results in substitution of one or more amino acids, but do not affect the functional properties of the protein encoded by the DNA sequence. The term "substantially similar" also refers to modifications of the nucleic acid fragments of the instant invention such as deletion or insertion of one or more nucleotide bases that do not substantially affect the functional properties of the resulting transcript.

In one aspect the invention also provides nucleic acids which are at least 60%, 70%, 80% 90%, 95%, 99%, or 99.5% identical to the nucleic acids disclosed herein.

The percentage of identity of two nucleic acid sequences or two amino acid sequences can be determined using the algorithm of Thompson et al. (CLUSTALW, 1994 Nucleic Acid Research 22: 4673-4, 680). A nucleotide sequence or an amino acid sequence can also be used as a so-called "query sequence" to perform a search against public nucleic acid or protein sequence databases in order, for example, to identify further unknown homologous sequences, which can also be used in embodiments of this invention. Such searches can be performed using the algorithm of Karlin and Altschul (1999 Proceedings of the National Academy of Sciences U.S.A. 87: 2,264 to 2,268), modified as in Karlin and Altschul (1993 Proceedings of the National Academy of Sciences U.S.A. 90: 5,873 to 5,877). Such an algorithm is incorporated in the NBLAST and XBLAST programs of Altschul et al. (1999 Journal of Molecular Biology 215: 403 to 410). Where gaps exist between two sequences, gapped BLAST can be utilized as described in Altschul et al. (1997 Nucleic Acid Research, 25: 3,389 to 3,402).

"Recombinant" refers to polynucleotides synthesized or otherwise manipulated in vitro ("recombinant polynucleotides") and to methods of using recombinant polynucleotides to produce gene products encoded by those polynucleotides in cells or other biological systems. For example, a cloned polynucleotide may be inserted into a suitable expression vector, such as a bacterial plasmid, and the plasmid can be used to transform a suitable host cell. A host cell that comprises the recombinant polynucleotide is referred to as a "recombinant host cell" or a "recombinant bacterium" or a "recombinant cyanobacteria." The gene is then expressed in the recombinant host cell to produce, e.g., a "recombinant protein." A recombinant polynucleotide may serve a non-coding function (e.g., promoter, origin of replication, ribosome-binding site, etc.) as well.

The term "transformation" is used herein to mean the insertion of heterologous or endogenous genetic material into the host cell via recombinant methods. Typically, the genetic material is DNA on a plasmid vector, but other means can also be employed. General transformation methods and selectable markers for bacteria and cyanobacteria are known in the art (Wirth, Mol Gen Genet. 216:175-177 (1989); Koksharova, Appl Microbiol Biotechnol 58:123-137 (2002). Additionally, transformation methods and selectable markers for use in bacteria are well known (see, e.g., Sambrook et al, supra).

The term "homologous recombination" refers to the process of recombination between two nucleic acid molecules based on nucleic acid sequence similarity. The term embraces both reciprocal and nonreciprocal recombination (also referred to as gene conversion). In addition, the recombination can be the result of equivalent or non-equivalent crossover events. Equivalent crossing over occurs between two equivalent sequences or chromosome regions, whereas nonequivalent crossing over occurs between identical (or substantially identical) segments of nonequivalent sequences or chromosome regions. Unequal crossing over typically results in gene duplications and deletions. For a description of the enzymes and mechanisms involved in homologous recombination see Court et al., "Genetic engineering using homologous recombination," Annual Review of Genetics 36:361-388; 2002.

The term "non-homologous or random integration" refers to any process by which DNA is integrated into the genome that does not involve homologous recombination. It appears to be a random process in which incorporation can occur at any of a large number of genomic locations.

The term "vector" as used herein is intended to refer to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid," which generally refers to a circular double stranded DNA molecule into which additional DNA segments may be ligated, but also includes linear double-stranded molecules such as those resulting from amplification by the polymerase chain reaction (PCR) or from treatment of a circular plasmid with a restriction enzyme.

Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., vectors having an origin of replication which functions in the host cell) such as extrachromosomal plasmids. Other vectors can be integrated into the genome of a host cell upon introduction into the host cell, and are thereby replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" (or simply "expression vectors").

The term "promoter" is intended to include a polynucleotide segment that can transcriptionally control a recombinant gene of interest, e.g., a pyruvate decarboxylase gene that it does or does not transcriptionally control in nature. In one embodiment, the transcriptional control of a promoter results in an increase in expression of the gene of interest. In an embodiment, a promoter is placed 5' to the gene-of-interest. A heterologous promoter can be used to replace the natural promoter, or can be used in addition to the natural promoter. A promoter can be endogenous with regard to the host cell in which it is used or it can be a heterologous polynucleotide sequence introduced into the host cell, e.g., exogenous with regard to the host cell in which it is used. Promoters of the invention may also be inducible, meaning that certain exogenous stimuli (e.g., nutrient starvation, heat shock, mechanical stress, light exposure, etc.) will induce the promoter leading to the transcription of the gene.

The phrase "operably linked" means that the nucleotide sequence of the nucleic acid molecule or gene of interest is linked to the regulatory sequence(s) in a manner which allows for regulation of expression (e.g., enhanced, increased, constitutive, basal, attenuated, decreased or repressed expression) of the nucleotide sequence and expression of a gene product encoded by the nucleotide sequence (e.g., when the recombinant nucleic acid molecule is included in a recombinant vector, as defined herein, and is introduced into a microorganism).

The term "gene" refers to an assembly of nucleotides that encode a polypeptide, and includes cDNA and genomic DNA nucleic acids. "Gene" also refers to a nucleic acid fragment that expresses a specific protein or polypeptide, including regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence.

The term "endogenous" refers to genes or genetic regulatory elements, such as promoters, which are present in the respective wild type cyanobacterial species. "Recombinant" genes or regulatory elements can also be included in non-natural recombinant plasmids within these cyanobacterial species or inserted into the genome of its native host cell via recombinant methods. In another embodiment of the invention "endogenous" also refers to genes or genetic elements, which are not present in the respective wild type cyanobacterial species, but which are present in other wild type species of the same genus, for example *Chlorogloeopsis*. Therefore the term "endogenous promoter" also can refer to a native promoter of *Chlorogloeopsis* PCC6912 recombinantly included in for example *Chlorogloeopsis* PCC9212. In this context, the inventors could show that some of promoters, for example the PziaA homologs can be identical between different *Chlorogloeopsis* species for example *Chlorogloeopsis* PCC6912 and *Chlorogloeopsis* PCC9212. In addition the promoters used in the present invention also might be at least 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99 percent identical or can be 100% identical to endogenous promoters. Any nucleotide changes in comparison to the native endogenous promoters can occur at least in one of the following regions:

The TATA box, and/or
the ribosomal binding side
the operator side and/or
the 5'-untranslated region (5'-UTR).

Additionally, the nucleotides between these functional regions can also be altered, deleted or additional nucleotides can be introduced.

A "foreign" gene or "heterologous" gene refers to a gene not normally found in the host organism, but that is introduced into the host organism by gene transfer. Foreign genes can comprise native genes inserted into a non-native organism, or chimeric genes. A "transgene" is a gene that has been introduced into the genome by a transformation procedure.

The term "fragment" refers to a nucleotide sequence of reduced length relative to the reference nucleic acid and comprising, over the common portion, a nucleotide sequence substantially identical to the reference nucleic acid. Such a nucleic acid fragment according to the invention may be, where appropriate, included in a larger polynucleotide of which it is a constituent. Such fragments comprise, or alternatively consist of, oligonucleotides ranging in length from at least about 6 to about 1,500 or more consecutive nucleotides of a polynucleotide according to the invention.

The term "open reading frame" abbreviated as "ORF," refers to a length of nucleic acid sequence, either DNA, cDNA or RNA, that comprises a translation start signal or initiation codon, such as an ATG or AUG, and a termination codon and can be potentially translated into a polypeptide sequence.

The term "upstream" refers to a nucleotide sequence that is located 5' to reference nucleotide sequence. In particular, upstream nucleotide sequences generally relate to sequences that are located on the 5' side of a coding sequence or starting point of transcription. For example, most promoters are located upstream of the start site of transcription.

The term "downstream" refers to a nucleotide sequence that is located 3' to a reference nucleotide sequence. In particular, downstream nucleotide sequences generally relate to sequences that follow the starting point of transcription. For example, the translation initiation codon of a gene is located downstream of the start site of transcription.

The terms "restriction endonuclease" and "restriction enzyme" refer to an enzyme that binds and cuts within a specific nucleotide sequence within double stranded DNA.

The term "expression" as used herein, refers to the transcription and stable accumulation mRNA derived from a nucleic acid or polynucleotide. Expression may also refer to translation of mRNA into a protein or polypeptide.

An "expression cassette" or "construct" refers to a series of polynucleotide elements that permit transcription of a gene in a host cell. Typically, the expression cassette includes a promoter and a heterologous or native polynucleotide sequence that is transcribed. Expression cassettes or constructs may also include, e.g., transcription termination signals, polyadenylation signals, and enhancer elements.

The term "codon" refers to a triplet of nucleotides coding for a single amino acid.

The term "codon-anticodon recognition" refers to the interaction between a codon on an mRNA molecule and the corresponding anticodon on a tRNA molecule.

The term "codon bias" refers to the fact that different organisms use different codon frequencies.

The term "codon improvement" refers to the modification of at least some of the codons present in a heterologous gene sequence from a triplet code that is not generally used in the host organism to a triplet code that is more common in the particular host organism. This can result in a higher expression level of the gene of interest. In particular, codon improvement or codon optimization can mean that the overall usage of the codons of a gene is adapted to more closely resemble or even be identical to the codon usage table of a certain organism, for example *Chlorogloeopsis fritschii* PCC6912.

The term "reporter gene" means a nucleic acid encoding an identifying factor that can be identified based upon the reporter gene's effect, in order to determine or confirm that a cell or organism contains the nucleic acid of interest, and/or to measure gene expression induction or transcription. Examples of reporter genes known and used in the art include but are not limited to luciferase (Luc), green fluorescent protein (GFP), chloramphenicol acetyltransferase (CAT), β-galactosidase (LacZ), β-glucuronidase (GUS), and the like. In embodiments of the present invention, recombinant genes coding for GFP can be included in the extrachromosomal plasmids harboring the ethanologenic cassettes so that the presence of the plasmids in the host cells can be detected easily via fluorescence. Selectable marker genes may also be considered reporter genes.

The term "selectable marker" means an identifying factor, usually an antibiotic or chemical resistance gene, that is able to be selected for based upon the marker gene's effect, such as resistance to an antibiotic, resistance to a herbicide, colorimetric markers, enzymes, fluorescent markers, and the like, wherein the effect is used to track the inheritance of a nucleic acid of interest and/or to identify a cell or organism that has inherited the nucleic acid of interest. Examples of selectable marker genes known and used in the art include: genes providing resistance to ampicillin, streptomycin, gentamycin, spectinomycin, kanamycin, hygromycin, neomycin and the like.

A "polypeptide" is a polymeric compound comprised of covalently linked amino acid residues. A "protein" is a polypeptide that performs a structural or functional role in a living cell.

A "heterologous protein" refers to a protein not naturally produced in the cell.

An "isolated polypeptide" or "isolated protein" is a polypeptide or protein that is substantially free of those compounds that are normally associated therewith in its natural state (e.g., other proteins or polypeptides, nucleic acids, carbohydrates, lipids).

The term "fragment" of a polypeptide refers to a polypeptide whose amino acid sequence is shorter than that of the reference polypeptide. Such fragments of a polypeptide according to the invention may have a length of at least about 2 to about 300 or more amino acids.

A "variant" of a polypeptide or protein is any analogue, fragment, derivative, or mutant which is derived from a polypeptide or protein and which retains at least one biological property of the polypeptide or protein. Different variants of the polypeptide or protein may exist in nature. These variants may be allelic variations characterized by differences in the nucleotide sequences of the structural gene coding for the protein, or may involve differential splicing or post-translational modification. The skilled artisan can produce variants having single or multiple amino acid substitutions, deletions, additions, or replacements.

As used herein, the phrase "increased activity" refers to any genetic modification resulting in increased levels of enzyme function in a host cell. As known to one of ordinary skill in the art, enzyme activity may be increased by increasing the level of transcription, either by modifying promoter function or by increasing gene copy number, increasing translational efficiency of an enzyme messenger RNA, e.g., by modifying ribosomal binding, or by increasing the stability of an enzyme, which increases the half-life of the protein, leading to the presence of more enzyme molecules in the cell. All of these represent non-limiting examples of increasing the activity of an enzyme. (mRNA Processing and Metabolism: Methods and Protocols, Edited by Daniel R. Schoenberg, Humana Press Inc., Totowa, N.J.; 2004; ISBN 1-59259-750-5; Prokaryotic Gene Expression (1999) Baumberg, S., Oxford University Press, ISBN 0199636036; The Biomedical Engineering Handbook (2000) Bronzino, J. D., Springer, ISBN 354066808X).

The terms "pyruvate decarboxylase" and "PDC" refer to an enzyme that catalyzes the decarboxylation of pyruvic acid to acetaldehyde and carbon dioxide. A "pdc gene" refers to the gene encoding an enzyme that catalyzes the decarboxylation of pyruvic acid to acetaldehyde and carbon dioxide.

The terms "Alcohol dehydrogenase" and "ADH" refer to an enzyme that catalyzes the interconversion between alcohols and aldehydes or ketones. An "adh gene" refers to the gene encoding an enzyme that catalyzes the interconversion between alcohols and aldehydes or ketones, "pdc/adh" refers to the pdc and adh genes collectively. A "pdc/adh cassette" refers to a nucleic acid sequence encoding a PDC enzyme and an Adh enzyme.

The term "primer" is an oligonucleotide that hybridizes to a target nucleic acid sequence to create a double stranded nucleic acid region that can serve as an initiation point for DNA synthesis under suitable conditions. Such primers may be used in a polymerase chain reaction.

Database entry numbers given in the following are from the NCBI database (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov) or from the CyanoBase, the genome database for cyanobacteria ((http://bacteria.ka-

zusa.orjp/cyanobase/index.html); Yazukazu et al. "Cyano-Base, the genome database for *Synechocystis* sp. Strain PCC6803: status for the year 2000", Nucleic Acid Research, 2000, Vol. 18, page 72).

The EC numbers cited throughout this patent application are enzyme commission numbers which is a numerical classification scheme for enzymes based on the chemical reactions which are catalyzed by the enzymes.

The *Chlorogloeopsis* sp. host cells and other cyanobacterial strains described in this patent application can be obtained from the Pasteur Culture Collection (PCC) of cyanobacteria, France, from the Culture Collection of Autotrophic Organisms (CCALA), Institute of Botany, Academy of Sciences of the Czech Republic, or were deposited by Algenol Biofuels Inc.

DESCRIPTION OF EMBODIMENTS OF THE INVENTION

One embodiment of the invention is directed to a genetically enhanced *Chlorogloeopsis* sp. host cell comprising at least one first recombinant gene encoding a first protein for the production of ethanol under the transcriptional control of a first inducible promoter, having at least 85%, 90% or 95% sequence identity to an endogenous inducible promoter of the *Chlorogloeopsis* sp. host cell.

The inventors of the present invention found that first inducible endogenous promoters for transcriptional control of at least the first recombinant gene for ethanol production of *Chlorogloeopsis* are important in order to establish a relatively high, constant and stable ethanol production in the *Chlorogloeopsis* sp. host cells for more than 3 weeks, preferably more than 5 weeks. Heterologous first promoters from different cyanobacterial genera, such as *Synechocystis* PCC6803 or *Nostoc/Anabaena* PCC7120 did not allow for a relatively high and stable, respectively ethanol production.

For example, the Zn^{2+} inducible PziaA promoter from *Synechocystis* PCC6803 was also found to be Zn^{2+} inducible in *Chlorogloeopsis* sp. host cells such as *Chlorogloeopsis* PCC9212, but resulted in very low ethanol production rates of 0.004-0.007% (v/v)/OD*d^{-1}, when controlling at least the transcription of the first recombinant gene encoding PDC enzyme so that this promoter was not used further in *Chlorogloeopsis* sp. host cells (see for example the below mentioned results for plasmid TK122). A second promoter PpetE from *Nostoc/Anabaena* PCC7120, which in its native host is Cu^{2+} responsive was shown to be a constitutive promoter if inserted directly upstream of a first recombinant gene for ethanol production such as a PDC enzyme encoding gene in an extrachromosomal plasmid transformed into *Chlorogloeopsis* sp. host cells (see for example the below results for plasmid TK18). This plasmid initially resulted in high ethanol production rates in *Chlorogloeopsis* sp. host cells, which however grew very slowly even during the upscaling process due to the constant ethanol production. After 2 to 3 weeks of cultivation ethanol production stopped, probably because the host cells reverted back to their wild-type.

In contrast to that, first promoters for transcriptionally controlling at least the first recombinant gene for ethanol production, which are inducible and endogenous to the *Chlorogloeopsis* sp. host cells were shown to enable a relatively high and constant ethanol production, which also could be maintained for at least three weeks or more.

The finding that endogenous inducible promoters are important for a successful ethanol production in *Chlorogloeopsis* sp. host cells is also not suggested by the prior art of Stucken et al., which shows that also constitutive heterologous promoters, for example the trc promoter from *E. coli* can be used for the recombinant overexpression of GFP protein in *Chlorogloeopsis*.

According to a further embodiment of the invention, the first inducible promoter can be a metal ion inducible promoter, especially a Zn^{2+}, or Co^{2+} inducible promoter such as the promoter PziaA from *Chlorogloeopsis fritschii* PCC6912, or the promoters controlling the open reading frame orf7041, orf7345, orf5189 and also orf5203 all from *Chlorogloeopsis* PCC6912. These endogenous metal ion inducible promoters can lead to relatively high and stable ethanol production rates (see for example the below experimental data showing higher ethanol production rates for plasmids TK187, TK261, TK414, TK336 and TK346 transformed into *Chlorogloeopsis* sp. host cells including either PziaA or Porf7041 from *Chlorogloeopsis fritschii* PCC6912 upon Zn^{2+}/Co^{2+} induction, and data showing some ethanol production for the plasmid TK351 including the Zn^{2+} inducible promoter from orf5189 from *Chlorogloeopsis fritschii* PCC6912).

In a further embodiment of the invention, the first endogenous inducible promoter can be generalized PziaA promoter with the following sequence:

N_{61}AACATCTGAATATATATTCAGATATTN_{1}TAAACTN_{26}**ACTGAAA**N_{5}**ATG** (SEQ ID NO: 36)

wherein the underlined sequence is the operator sequence, the boxed sequence is the TATA box and the underlined boldfaced sequence is the ribosomal binding site and wherein each of the nucleotides N is independently selected from a group consisting of A, T, C and G and wherein the 3'-ATG is the start codon for the first recombinant gene transcriptionally controlled by this promoter.

Furthermore, the *Chlorogloeopsis* sp. host cell can be a host cell from the well established strains *Chlorogloeopsis fritschii* PCC6912, *Chlorogloeopsis* PCC9212 or *Chlorogloeopsis* sp. ABICyano3.

A deposit of the Algenol Biofuels Inc. proprietary strain of *Chlorogloeopsis* sp. ABICyano3, disclosed in the present application and recited in the appended claims has been made with the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, Va. 20110. The date of deposit was Oct. 10, 2013. The ATCC Accession Number is PTA-120619.

Preferably the *Chlorogloeopsis* sp. host cell is *Chlorogloeopsis fritschii* PCC6912. This cyanobacterial strain can produce ethanol in brackish water in ranges of salinity between 6 to 20 psu, preferably 8.75 to 17.5 psu. Furthermore *Chlorogloeopsis* PCC6912 can also form aggregates during cultivation which sink rapidly to the bottom of the photobioreactors, when mixing by for example stirring or bubbling is stopped, so that old medium can easily be removed from the upper parts of the medium having a lower concentration of the *Chlorogloeopsis* sp. host cells. Following this procedure ensures that not too many *Chlorogloeopsis fritschii* PCC6912 cells are removed by exchanging parts of the medium. In addition ethanol can easily be removed from top parts of the medium having a lower concentration of *Chlorogloeopsis fritschii* PCC6912 cells.

In addition, the *Chlorogloeopsis* sp. host cells can show high ethanol production rates of at least 0.01%(v/v) d^{-1}, preferably at least 0.025%(v/v) d^{-1}, most preferred at least 0.028% (v/v) d^{-1} and up to at least 0.05%(v/v) d^{-1} for at least 7 days of cultivation (see FIG. **7**D for the single cultivation). The accumulated ethanol production can reach over 0.4% (v/v) over 14 days of cultivation in BG11 medium corresponding to a production rate of 0.033% (v/v) d^{-1} (see FIG. **7**B).

The ethanol production of *Chlorogloeopsis fritschii* PCC6912 e.g. in brackish media with a salinity of between 8.7 and 17.5 psu resulted in about 0.36 to 0.39% (v/v) ethanol after 23 days (FIG. **17**A) of cultivation and is higher compared to the ethanol production rate of the other *Chlorogloeopsis* sp. host cells, such as *Chlorogloeopsis* sp. PCC9212, in brackish media.

In a further variant of the invention, the *Chlorogloeopsis* sp. host cell can also comprise at least one second recombinant gene encoding a second protein for the production of ethanol in addition to the first recombinant gene for ethanol production. For example the first recombinant gene can encode pyruvate decarboxylase converting pyruvate into acetaldehyde and the second recombinant gene can encode alcohol dehydrogenase further converting acetaldehyde into ethanol.

Alternatively or in addition, the first recombinant gene can also encode alcohol dehydrogenase E enzyme (AdhE enzyme) which can directly convert acetyl-Coenzyme A into ethanol. Genes encoding alcohol dehydrogenase E are for example disclosed in the PCT application WO 2009/098089 A2, which is incorporated for this purpose.

In this context, it is possible that the same first endogenous inducible promoter controls the transcription of both the first and second recombinant gene. This is for example the case in one of the plasmids which enable a high ethanol production rate in *Chlorogloeopsis* sp. host cells, for example the plasmid TK187.

Furthermore, it is also possible that the first and second recombinant genes are under the transcriptional control of separate first and second promoters. In this case it might be preferred if the induction mechanism of the second promoter is different from the first endogenous inducible promoter, i. e. either the second promoter is inducible by a different inductor in comparison to the first promoter or the second promoter is a constitutive promoter. In this case the second recombinant gene, for example the alcohol dehydrogenase, is permanently produced during the scale up and cultivation of the *Chlorogloeopsis* sp. host cells, so that the harmful acetaldehyde can be quickly converted to the less toxic ethanol once the first recombinant gene, for example pyruvate decarboxylase is produced in high quantities, when the first promoter is induced.

Alternatively, the second promoter can also be an inducible promoter, which can be induced by different inductors, for example other metal ions in comparison to the first inducible endogenous promoter.

In the case that the second promoter is a constitutive promoter it can be selected from a group consisting of PpetE, or PnblA from *Nostoc/Anabaena* PCC7120, which are used in many of the plasmids disclosed herein. The plasmid TK336 used herein contains the first recombinant gene encoding PDC enzyme under the control of the first Zn^{2+} inducible PziaA promoter, whereas the second recombinant gene coding for alcohol dehydrogenase is under the control of the promoter PnblA from *Nostoc/Anabaena* PCC7120, which was shown to be a constitutive promoter in the *Chlorogloeopsis* PCC6912 host cells.

In addition, the at least one first and also if present the at least one second recombinant gene can be codon improved for enhancing translation by having a codon adaptation index of equal to or greater than 0.6, preferably equal to or greater than 0.7 most preferred greater than or equal to 0.8 based on the below codon usage table of the *Chlorogloeopsis* sp. host cell.

TABLE 1

Codon Usage
Codon Usage table

Amino Acid	Codon	Number	/1000	Fraction
Ala	GCG	21513.00	10.38	0.13
Ala	GCA	56949.00	27.49	0.33
Ala	GCT	60693.00	29.30	0.36
Ala	GCC	30886.00	14.91	0.18
Cys	TGT	12937.00	6.24	0.59
Cys	TGC	8898.00	4.30	0.41
Asp	GAT	71751.00	34.63	0.75
Asp	GAC	23767.00	11.47	0.25
Glu	GAG	35182.00	16.98	0.27
Glu	GAA	94948.00	45.83	0.73
Phe	TTT	64339.00	31.06	0.76
Phe	TTC	19940.00	9.62	0.24
Gly	GGG	17992.00	8.68	0.13
Gly	GGA	37399.00	18.05	0.28
Gly	GGT	50906.00	24.57	0.38
Gly	GGC	27994.00	13.51	0.21
His	CAT	22815.00	11.01	0.58
His	CAC	16302.00	7.87	0.42
Ile	ATA	23782.00	11.48	0.17
Ile	ATT	82747.00	39.94	0.59
Ile	ATC	34003.00	16.41	0.24
Lys	AAG	28873.00	13.94	0.29
Lys	AAA	72057.00	34.78	0.71
Leu	TTG	52946.00	25.56	0.23
Leu	TTA	67444.00	32.56	0.30
Leu	CTG	29147.00	14.07	0.13
Leu	CTA	29246.00	14.12	0.13
Leu	CTT	27727.00	13.38	0.12
Leu	CTC	20752.00	10.02	0.09
Met	ATG	38148.00	18.41	1.00
Asn	AAT	58913.00	28.44	0.66
Asn	AAC	30157.00	14.56	0.34
Pro	CCG	11772.00	5.68	0.12
Pro	CCA	33050.00	15.95	0.34
Pro	CCT	31169.00	15.05	0.32
Pro	CCC	20628.00	9.96	0.21
Gln	CAG	32669.00	15.77	0.29
Gln	CAA	78214.00	37.75	0.71
Arg	AGG	6979.00	3.37	0.06
Arg	AGA	17363.00	8.38	0.16
Arg	CGG	12623.00	6.09	0.12
Arg	CGA	17024.00	8.22	0.16
Arg	CGT	24665.00	11.91	0.23
Arg	CGC	28950.00	13.97	0.27
Ser	AGT	30130.00	14.54	0.23
Ser	AGC	21582.00	10.42	0.17
Ser	TCG	10220.00	4.93	0.08
Ser	TCA	20353.00	9.82	0.16
Ser	TCT	33178.00	16.01	0.26
Ser	TCC	12875.00	6.21	0.10
Thr	ACG	11429.00	5.52	0.10
Thr	ACA	37859.00	18.27	0.33
Thr	ACT	43814.00	21.15	0.38
Thr	ACC	22203.00	10.72	0.19
Val	GTG	29904.00	14.43	0.21
Val	GTA	41715.00	20.14	0.30
Val	GTT	48038.00	23.19	0.34
Val	GTC	20305.00	9.80	0.15
Trp	TGG	30046.00	14.50	1.00
Tyr	TAT	39044.00	18.85	0.61
Tyr	TAC	25181.00	12.15	0.39
End	TGA	1668.00	0.81	0.22
End	TAG	2076.00	1.00	0.27
End	TAA	3815.00	1.84	0.50

The column titled "/1000" shows the frequency of the respective codon per 1000 bases of a coding DNA sequence in *Chlorogloeopsis* PCC6912. The column titled "number" denotes the overall number of the respective codon in the genome of *Chlorogloeopsis fritschii* PCC6912. The column titled "fraction" denotes the fractional amount of one codon coding for a particular amino acid in relation to the fractional amounts of the other codons coding for the same amino acid. The sum of all fractional amounts of the codons for one amino acid is 1.

It might be advantageous to include a transcription terminator between the first and second recombinant gene in order to disconnect the transcriptional control of the first and second recombinant gene as for example shown in the plasmid TK336 where the oop terminator is present between the first and second recombinant gene if the first recombinant gene and the second recombinant gene are controlled by different first and second promoters.

The first and if present second recombinant genes including their promoters can be either located on an extrachromosomal plasmid or can be integrated into a chromosome of the *Chlorogloeopsis* sp. host cell.

Furthermore, the inventors realized that extrachromosomal plasmids containing the ethanologenic cassettes need to contain an origin of replication from a closely related species such as *Nostoc/Anabaena*, for example the origin of replication pDU1 (the protein sequence of the replication protein of the pDU1 plasmid from *Nostoc* sp. PCC 7524 is shown as SEQ ID NO. 15, the protein sequence of the integrase/resolvase recombinase from *Nostoc* sp. PCC 7524 is shown in SEQ ID NO. 16, and the nucleic acid sequence of the respective origin of replication is included in the sequence listing as SEQ ID NO. 17) in order to independently replicate in the *Chlorogloeopsis* sp. host cells. Extrachromosomal plasmids based on a different origin of replication such as the origin of replication of RSF1010 such as pVZ321 did not result in genetically enhanced *Chlorogloeopsis* sp. host cells.

The *Chlorogloeopsis* sp. host cells were found to tolerate very harsh culturing conditions concerning both the concentration of ethanol in the medium as well as the range of temperature for cultivation and oxygen stress. In particular, the *Chlorogloeopsis* sp. host cells can withstand at least 1% (v/v) ethanol in the culture medium for at least 6, 12, 16 and up to 27 weeks. Furthermore the cells can withstand at least 48° C., preferably at least 50° C. or at least 53 to 55° C. for at least 2 hours peaks over at least 7 days in brackish media up to 15 to 17 psu. *Chlorogloeopsis* PCC 6912 can also withstand a purging of the culture medium with 60 to 70% oxygen.

The test for ethanol tolerance was performed by adding 1% ethanol to the medium of the *Chlorogloeopsis* sp. host cells. Additional ethanol was added throughout cultivation, in the case that the ethanol level decreased in order to keep the ethanol level at 1%. Cyanobacterial cultures were then examined for example under the microscope after a pre-determined period of time for example 6, 12 or 16 weeks and cyanobacterial cultures were deemed to have passed the ethanol tolerance test if at least more than 50% of the cyanobacterial cells were found to be intact and viable according to microscopic analysis, meaning that the cell morphology did not change significantly; the cells were still green and the cells were not lysed.

The test for temperature tolerance was conducted with the *Chlorogloeopsis* sp. host cells in a medium under conditions of light illumination and omitting light illumination (day/night cycle) at maximum temperatures between 45 to 55° C. for a certain period of time, for example 1 to 2 hours during illumination. Cyanobacterial cells were deemed to have passed the test if the cultures were still growing after having been subjected to 7 days of day/night cycles as described above. Growth could be detected for example by an increase in the chlorophyll content of the cyanobacterial cultures. *Chlorogloeopsis fritschii* PCC6912 for example was found to withstand 48° C., 50° C. or at least 53 to 55° C. for at least 2 hours per day over a time period of at least 7 days even in medium with a salinity of 35 psu.

In addition, an oxygen tolerance test was carried out which showed that *Chlorogloeopsis fritschii* PCC6912 can tolerate purging of the medium with 60% to 70% oxygen resulting an oxygen levels of up to 650 μmol/l in cultures during the day, when cultured a temperatures between 28° C. to 37° C. and then being illuminated with a light intensity of between 200 $\mu E \times m^{-2} \times s^{-1}$ to 400 $\mu E \times m^{-2} \times s^{-1}$.

The results for the ethanol tolerance tests and temperature tolerance tests for various different cyanobacterial strains including the *Chlorogloeopsis* sp. host cells of the present invention are shown in the following Table 2:

TABLE 2

Strain Characterization							
	Growth in marine	1% EtOH tolerance	Thermotolerance test (each test for 1 week)			Additional characterization (each test for 1 week)	
Cyanobacterial species	medium (35 psu)	test [weeks]	2 hours 45° C.	2 hours 48° C.	2 hours 50° C.	2 hours 53° C.	2 hours 55° C.
Chlorogloeopsis ABICyano3	pos.	>11 (in (marine BG11 30 psu)	pos. (up to 27 psu)	pos. (up to 27 psu)	pos. (up to 27 psu)	pos. (up to 27 psu)	pos. (up to 27 psu)
Chlorogloeopsis PCC6912	pos.	27 (in (marine BG11 30 psu)	pos. (35 psu)	pos. (35 psu)	pos. (35 psu)	pos. (35 psu)	pos. (35 psu)
Chlorogloeopsis PCC9212	neg.	11*	pos. (7.5 psu)	pos. (15 psu)	pos. (7.5 psu)	pos. (7.5 psu)	pos. 7.5 psu)
Thermosynechococcus elongates BP-1	neg.	<1	pos. (BG11)	pos. (BG11)	n.d.	pos. (BG11)	pos. (BG11)
Chroococcidiopsis thermalis	pos.	3	n.d.	n.d.	n.d.	n.d.	n.d.

TABLE 2-continued

Strain Characterization							
	Growth in marine	1% EtOH tolerance	Thermotolerance test (each test for 1 week)			Additional characterization (each test for 1 week)	
Cyanobacterial species	medium (35 psu)	test [weeks]	2 hours 45° C.	2 hours 48° C.	2 hours 50° C.	2 hours 53° C.	2 hours 55° C.
CCALA187 *Chroococcidiopsis thermalis*	pos.	3	n.d.	n.d.	n.d.	n.d.	n.d.
CCALA50 *Chroococcidiopsis thermalis* CCALA48	pos.	3	pos. (Z)	pos. (Z)	neg. (Z)		

Pos. = positive
Neg. = negative
N.d. = Not determined
Z = Z medium
* = test was done in BG11 medium

The table clearly shows that *Chlorogloeopsis* PCC6912 appears to be the most salt and ethanol tolerant cyanobacterial strain in the table and can withstand 1% ethanol in marine BG 11 medium for 27 weeks, whereas other thermotolerant strains such as *Chroococcidiopsis thermalis* can only tolerate 1% ethanol in BG11 medium for three weeks. The two other *Chlorogloeopsis* strains PCC9212 and ABICyano3 are less salt tolerant compared to PCC6912 in the thermotolerance test, because they can only withstand the same high temperatures as PCC6912 in media with lower salt concentration. *Chlorogloeopsis* PCC6912 was also the most sturdy strain in the thermotolerance test because it was able to tolerate two hours at 45° C. for one week, two hours 48° C. for one week and also two hours at 50° C. for one week in marine medium (35 psu).

Another aspect of the present invention is directed to a method for producing ethanol comprising the method steps of:

A) culturing any of the above described genetically enhanced *Chlorogloeopsis* sp. host cells in a culture medium, the host cells thereby producing ethanol,

B) retrieving ethanol from at least either one of the host cells, the medium or the head space above the medium.

Due to the sturdiness of the *Chlorogloeopsis* sp. host cells during method step A) the host cells can be cultured under at least one of the following culturing conditions:

Temperatures between 20° C. to about 55° C., preferably between 25° C. to 45° C. and/or a salinity of the culture medium of between 0.2 to 35 psu, in particular between 8.7 to 17.5 psu. In particular, the *Chlorogloeopsis fritschii* PCC6912 host cells can therefore also be cultivated in brackish medium. This finding is particularly surprising, because *Chlorogloeopsis fritschii* PCC6912 is known to be a freshwater strain.

Chlorogloeopsis sp. host cells can tolerate a wide range of temperatures, for example moderate temperatures of around 20° C. to more extreme temperatures of around 55° C., so that these cells can easily be cultivated under desert-like conditions, where during daytime high temperatures can be reached and during night time much lower temperatures can be expected.

Since the *Chlorogloeopsis* sp. host cells can form nitrogen fixing heterocysts, the culture medium does not need to include nitrogen as a source for growth, which is a clear advantage to the culturing of other cyanobacterial strains which require nitrogen for growth such as *Synechococcus* or *Synechocystis*.

In the case that *Chlorogloeopsis fritschii* PCC6912 cells or ABICyano3 cells are cultured, a mixing of the culture medium during the method step A), for example via stirring or via introducing gases from the bottom of the bioreactor, can be advantageous, in order to avoid a settlement of the cells at the bottom of the photobioreactor.

Without stirring, during the cultivation method step A), a larger fraction of the *Chlorogloeopsis fritschii* PCC6912 cells and ABICyano3 cells settle in bottom sections of the culture medium compared to a smaller fraction of the host cells being located in top sections of the culture medium. If a settlement of the cells occur, one of the following method steps can be performed very easily:

During method step A):

culture medium in the top section is removed and fresh culture medium is added

Chlorogloeopsis sp. host cells are removed from the bottom section of the culture medium and/or

During method step B):

ethanol is harvested in the top section of the culture medium.

Chlorogloeopsis PCC6912 and ABICyano3 form aggregates during cultivation; if mixing is stopped, those aggregates t_{end} to settle more on the bottom of the photobioreactor, so that for example from the top sections of the culture medium, used medium can be removed and can be replenished by new medium, thereby easing the whole cultivation procedure. With reference to FIG. **19**A, the cultivation can be done in vessels, for example bioreactors (3) harboring the culture medium (2) and containing an enclosed headspace (25) above the culture medium (2), including gases such as carbon dioxide and air or evaporated ethanol, which can be removed via the pipe (35). The top section of the culture medium is in contact with the headspace (25) and the bottom section of the culture medium is located below the top section. The top sections of the culture medium can be removed for example by using plug valves (10 and 20) located in the top sections of the photobioreactor for pumping the medium out of the photobioreactor by opening the valve. As long as gases (5), such as air or carbon dioxide are introduced from the bottom of the photobioreactor into the medium via a supply pipe (30) as shown in FIG. **19**A, the PCC6912 or ABICyano3 cells are evenly distributed in the medium in aggregates (1).

Once the bubbling of gases is discontinued as shown in FIG. **19**B (crossed out supply pipe 30), the cells settle in the bottom section (45) if allowed to settle for a period of time between 5 minutes to 60 minutes, at most 120 minutes. In contrast to the bottom section (45), the top section (40) is nearly free of the cells (less than 5% of the total cell mass of the cells, preferably less than 1% of the total mass of the cells would be located in the top section after settlement of the cells). Ethanol can now be more easily harvested from the top section (40) using for example the plug valves (10 and/or 20) so that the purification of ethanol is simplified. Removing more concentrated biomass of the *Chlorogloeopsis* sp. host cells from the bottom section (45) of the culture medium via for example the plug valve (15) is also easier.

Since most of the cells have settled in the bottom section, centrifuging the medium of the top section, which is normally done in order to remove the cells from the medium, is not necessary. The final separation of the ethanol from the medium of the top section can, for example, be done by vapor compression steam stripping as described in the PCT patent application WO 2011/103277 A1, which is hereby incorporated with regard to the separation procedure or via distillation.

In particular, the separation of the ethanol from the culture medium can be done via steam stripping process described in WO 2011/103277 A1, in which the culture medium, containing the ethanol is a dilute feed solution, the steam stripping process comprising the following method steps:

(a) counter-current contacting of the dilute feed solution and a vapor phase with a counter-current vapor-liquid contactor;

(b) condensing in a condensor of the vapor phase output of the counter-current vapor-liquid contactor with transfer of the latent heat released by condensation to an evaporator;

(c) evaporating of a liquid feed to provide the vapor phase input to the counter-current contactor; and

(d) compressing of the vapor phase, wherein compression of the vapor phase may occur before the contactor, after the contactor, or both before and after and wherein the action of the compressor must result in a pressure that is higher in the condenser than in the evaporator.

The vapor phase can be saturated with water and strips off the ethanol from the dilute feed solution upon counter-current contacting, resulting in a vapor phase enriched with ethanol, which then can be condensed.

Long-term cultivation of the *Chlorogloeopsis* PCC6912 and ABICyano3 cells can therefore be maintained by alternating between two modes of operation, mixing or discontinue mixing so that the cells settle. During the mixing, the cell cultures can grow and can therefore produce ethanol, whereas if mixing is stopped, either medium can be replenished, new cells can be introduced into the cell culture or the ethanol can be harvested very easily as described above. Afterwards mixing can be resumed.

In the case that the first endogenous inducible promoter is a metal ion inducible promoter, method step A) can include the substeps of:

A1) culturing the *Chlorogloeopsis* sp. host cells in an uninduced state, and the further method step of

A2) inducing the *Chlorogloeopsis* sp. host cells by adding metal ions to the culture medium.

This method is very simple and can easily be used for a reliable induction procedure. During the uninduced state, the *Chlorogloeopsis* sp. host cells can grow quickly so that the upscaling can easily be achieved.

Another aspect of the invention is directed to a method of producing the genetically enhanced *Chlorogloeopsis* sp. host cells comprising introducing said first and if present said second recombinant gene into the host cell. In general, said first and if present said second recombinant gene can be either be introduced into the chromosome of the host cell, into endogenous plasmids or can be introduced into the cell as a part of a heterologous extrachromosomal plasmid.

In particular the following method steps can be included in such a method:

a) Providing a recombinant nucleic acid sequence including said first and if present second recombinant gene and protecting said recombinant nucleic acid sequence against endogenous restriction endonucleases of the host cells, and the further method step of:

b) Introducing said first if present second recombinant gene into the genome of the host cell.

Such a protection step can for example be performed by methylating the plasmids for transformation of the *Chlorogloeopsis* sp. host cells using certain methylases in order to mask the specific restriction sites for the restriction endonucleases. Specific testing of the *Chlorogloeopsis* sp. host cells, especially the three strains *Chlorogloeopsis fritschii* sp. PCC6912, *Chlorogloeopsis* sp. PCC9212, and *Chlorogloeopsis* sp. ABICyano3 for restriction endonucleases, provided evidence that the restriction enzymes SphI and BlpI are present. Therefore, the methylases M. CviPI and M. SssI (New England Biolabs) can be used in order to protect the restriction sites against the action of these enzymes by in vitro methylation.

Alternatively, the recombinant nucleic acid sequence can be protected against endogenous restriction endonucleases by deleting and/or altering the specific recognition sequences of the endonucleases for example by in vitro gene synthesis.

Furthermore, during method step b) electroporation or conjugation can be used, preferably electroporation.

It was shown that especially *Chlorogloeopsis* PCC6912 host cells include a capsule or an extracellular polymer layer (EPS), which often can hinder an introduction of recombinant nucleic acids into the host cell. In the present case, however, the inventors found that neither sonification of the host cells nor incubation with a salt solution for at least one hour was necessary in order to enable a successful introduction of recombinant nucleic acids such as plasmids via conjugation or electroporation into the *Chlorogloeopsis* sp. host cells. This finding is also in clear contrast to the prior art document Stucken et al., which describes that sonification and a salt wash were critical steps for successful conjugation.

Another embodiment of the invention is directed to a construct for transformation of *Chlorogloeopsis* sp. host cells comprising:

at least one first recombinant gene encoding a first protein for the production of ethanol under the transcriptional control of a first inducible promoter, having at least 85%, 90% or 95% sequence identity to an endogenous inducible promoter of the *Chlorogloeopsis* sp. host cell.

Such a construct is well suited in order to produce genetically enhanced *Chlorogloeopsis* sp. host cells for ethanol production using the transformation protocols as described in this patent application. In particular, the endogenous promoter of the *Chlorogloeopsis* sp. host cells enables a stable and high ethanol production as described above.

The construct can be a plasmid, for example an extrachromosomal plasmid including an origin of replication for replication of the construct independently of the genome of the *Chlorogloeopsis* sp. host cells. Alternatively, the construct can also be an integrative plasmid containing DNA sequences

homologous to genomic sequences of the host cell for integration of a recombinant region flanked by these homologous regions into the chromosomes of the host cell. The recombinant region can include an ethanologenic cassette with said first recombinant gene and if necessary also antibiotic resistance conferring genes.

The first inducible promoter can be a metal-ion inducible promoter, especially a Zn^{2+}, or Co^{2+} inducible promoter as already described above.

The recombinant construct can also further include all the features already described with regard to the genetically enhanced *Chlorogloeopsis* sp. host cells, such as second recombinant gene for ethanol production and also the various different promoters.

EXAMPLES

Example 1

Bacterial Strains, Growth Conditions, and Selection of Transformants

Escherichia coli strains J53, HB101 (Promega), XL10-Gold (Stratagene), and α-select (Bioline) were grown in Luria-Bertani (LB) medium at 37° C. Ampicillin (50 μg/ml), kanamycin (25-50 μg/ml), and chloramphenicol (34 μg/ml) were used when appropriate. *E. coli* cultures were continuously shaken overnight at 200 rpm and at 100 rpm, respectively, when used for conjugation.

For transformation experiments cyanobacterial wild-type axenic strains were cultured at 28-35° C. in liquid BG11 fresh water on a reciprocal shaker at 150 rpm under continuous illumination of approximately 30-40 μmol photons/m^2 s.

Chlorogloeopsis transformants (derived from *Chlorogloeopsis fritschii* PCC6912, *Chlorogloeopsis* sp. PCC 9212, and *Chlorogloeopsis* sp. ABICyano3) were maintained on solid BG11 medium containing 25-50 μg/ml neomycin.

The liquid culture medium, BG11 and artificial seawater BG11 (aswBG11) for culturing either the wildtype or the genetically enhanced *Chlorogloeopsis* sp. host cells can be prepared as follows:

TABLE 3

Composition of BG-11 medium

Compound	Amount (per liter)	Final Concentration
$NaNO_3$	1.5 g	17.6 mM
K_2HPO_4	0.04 g	0.23 mM
$MgSO_4 \cdot 7H_2O$	0.075 g	0.3 mM
$CaCl_2 \cdot 2H_2O$	0.036 g	0.24 mM
Citric acid	0.006 g	0.031 mM
Ferric ammonium citrate	0.006 g	—
EDTA (disodium salt)	0.001 g	0.0030 mM
$NaCO_3$	0.02 g	0.19 mM
Trace metal mix A5	1.0 ml	—

TABLE 4

1000× Trace Metal Composition of BG-11 medium

1000× Trace Metal mix A5	Amount	Final Concentration in Working Medium
H_3BO_3	2.86 g	46.26 μM
$MnCl_2 \cdot 4H_2O$	1.81 g	9.15 μM
$ZnSO_4 \cdot 7H_2O$	0.222 g	0.772 μM
$NaMoO_4 \cdot 2H_2O$	0.39 g	1.61 μM
$CuSO_4 \cdot 5H_2O$	0.079 g	0.32 μM
$Co(NO_3)_2 \cdot 6H_2O$	49.4 mg	0.170 μM
Distilled water	1.0 L	—

Distilled water for BG11 or seawater (35 practical salinity units=psu; see Unesco (1981a). The Practical Salinity Scale 1978 and the International Equation of State of Seawater 1980. Tech. Pap. Mar. Sci., 36: 25 pp.) for mBG11 is added to the final volume of 1.0 L.

TABLE 5

Recipe for a 100× BG11 stock solution

100× BG11	g/L	mL/L
Sodium nitrate, waterfree ($NaNO_3$)	149.58	—
Magnesium sulfate -heptahydrate ($MgSO_4 \cdot 7H_2O$)	7.49	—
Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$)	3.6	—
Citric Acid	0.6	—
0.25M EDTA disodiumsalt dihydrate ($Na_2EDTA \cdot 2H_2O$ (pH 8.0))	—	1.12

TABLE 6

Recipe for artificial seawater aswBG-11 (35 psu)

35 psu artificial marine BG11	g/L	mL/L
Sodium chloride	25.84	—
Magnesium sulfate-heptahydrate	6.36	—
Magnesium(II)chloride	5.06	—
Potassium chloride	0.62	—
Calcium chloride dihydrate	1.36	—
100× BG11	—	10
20 mg/mL Disodium carbonate (Na_2CO_3)	—	1
40 mg/mL Dipotassium hydrogen phosphate trihydrate ($K_2HPO_4 \cdot 3H_2O$)	—	1

Use de-ionized water for preparing the solutions. Autoclave for 20 min at 121° C. After the media is cooled down add 1 mL 6 mg/mL Ferric ammonium citrate and 1 mL of the 1,000× trace metal mix.

TABLE 7

Recipe for Z media (from "Algal Culturing Techniques" by Academic Press, 2005)

Z media	mL/L L
1.1 mol/L $NaNO_3$	5
0.25 mol/L $Ca(NO_3)_2$	1
0.178 mol/L K_2HPO_4	1
0.1 mol/L $MgSO_4$	1
0.2 mol/L Na_2CO_3	1
10 mmol L EDTA-Na_2 and $FeCl_3$ 0, 1N HCl	1
Gaffron micronutrients	0.08

Use de-ionized water for media preparation. Autoclave for 20 min at 121° C.

TABLE 8

Recipe for Gaffron Nutrient Stock Solution

Stock solutions Gaffron micro_nutrients	g/L
H_3BO_3	3.100
$MnSO_4 \cdot 4H_2O$	2.230
$ZnSO_4 \cdot 7H_2O$	0.220
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.088
$Co(NO_3)_2 \cdot 6H_2O$	0.146
$VOSO_4 \cdot 6H_2O$	0.054
$Al_2(SO_4)_3K_2SO_4 \cdot 2H_2O$	0.474
$NiSO_4(NH_4)_2SO_4 \cdot 6H_2O$	0.198
$Cd(NO_3)_2 \cdot 4H_2O$	0.154
$Cr(NO_3)_3 \cdot 7H_2O$	0.037
$Na_2WO_4 \cdot 2H_2O$	0.033
KBr	0.119
KI	0.083

Example 2

Detection of a Capsule or an Extracellular Polymer Layer (EPS) Around *Chlorogloeopsis Fritschii* PCC6912

The procedure is based on the so called ConA-FITC fluorescence microscopy (Lectin-Fluorescein isothiocyanate conjugate from *Canavalia ensiformis*), which can be used for labelling of carbohydrate moieties on the cell surface of the cyanobacterial cells.

In particular, the cells were incubated in 1/1000 ConA/FITC (Sigma) for 30 min. Lectin was diluted in 50 mM phosphate buffer (pH 7.0), which contained 5 mM $MnCl_2$ and $CaCl_2$ ("lectin buffer"). The stained cells were then microscopically investigated with a fluorescence microscope. If FITC results in a high background fluorescence, cells were directly washed on the slide with a bit "lectin buffer" and extra volume sucked off with a tissue.

FIG. **1** shows a fluorescence photograph of *Chlorogloeopsis fritschii* PCC6912 cells labeled with ConA-FITC. The extracellular capsule or EPS appears in a different color than the cyanobacterial cells itself (red fluorescence for the cells and green fluorescence for the capsule or EPS).

Example 3

Detection of Cyanobacterial Restriction Endonucleases (RENs)

Restriction analysis was performed using different plasmids, which were incubated with crude extracts of the *Chlorogloeopsis* strains. 600 ng of plasmids K230, K236Cm and K244, respectively (the DNA sequences of plasmid K230, K236Cm and K244, respectively are shown as SEQ ID Nos. 18, 19 and 20), were incubated in NEB buffer P4 with ~13 μg total protein over night at 28° C. in 40 μl reaction volume and analyzed by agarose gel electrophoreses. Digestion bands were indicative of specific RENs present in the crude extract. Smearing indicated unspecific nucleolytic activity. Sequencing of digested plasmids led to identification of respective restriction sites.

K230 was column purified after incubation in crude extract and fragments were sent to sequencing with primers #417 (230spannA) to #434 (230spannR) covering the whole plasmid (the DNA sequences of both primers #417 and #434 are included in the sequence listing as SEQ ID NOs. 22 and 23). A sharp signal drop down—which required complete digestion—in the obtained sequence was indicative of a restriction point.

FIG. **2**A and FIG. **2**B show agarose gels evidencing the results of the incubation of the above mentioned plasmids with the crude extracts. A set of plasmids covering multiple different REN sites were incubated with crude extracts to identify RENs in the *Chlorogloeopsis* host cells. The lanes denote the following:

L: ladder, Lane 1: K230, lane 2: K236, lane 3: K244, lane 4: K230 plus *Chlorogloeopsis* sp. ABICyano3 crude extract, lane 5: K230 plus *Chlorogloeopsis fritschii* PCC6912 crude extract, lane 6: K230 plus *Chlorogloeopsis* sp. PCC9212 crude extract, lane 7: K236 plus *Chlorogloeopsis* sp. ABICyano3 crude extract, lane 8: K236 plus *Chlorogloeopsis fritschii* PCC6912 crude extract, lane 9: K236 plus *Chlorogloeopsis* sp. PCC9212 crude extract, lane 10: K244 plus *Chlorogloeopsis* sp. ABICyano3 crude extract, lane 11: K244 plus *Chlorogloeopsis fritschii* PCC6912 crude extract, lane 12: K244 plus *Chlorogloeopsis* sp. PCC9212 crude extract, lane 13: crude extract from *Chlorogloeopsis* sp. ABICyano3, lane 14: crude extract from *Chlorogloeopsis fritschii* PCC6912, lane 15: crude extract from *Chlorogloeopsis* sp. PCC9212. These gels clearly show that specific restriction pattern can be identified upon digestion of the plasmids with the three different cell extracts. Subsequent sequencing of these digested DNA patterns revealed the presence of the restriction endonucleases SphI and BlpI.

Example 4

Transformation of Cyanobacterial Strains

Gene transfer to *Chlorogloeopsis* strains was performed by conjugation and electroporation, respectively. Prior to electroporation DNA was protected by in vitro methylation using the commercially available methylates M. CviPI and M. SssI (New England Biolabs). In general, the transformation of the *Chlorogloeopsis* strains can be done in the same way using either extrachromosomal or integrative plasmids.

Methylations were conducted in 1× methylation buffer with 160-640 μM S-adenosylmethionine and methylase (1-5 U M.CviPI and/or 5 U M.SssI per 1 μg plasmid) for at least 4 hours at 37° C. Then, methylated DNA was extracted by phenol-chloroform-isoamylalcohol and precipitated by ethanol. After DNA pellet was dried at 65° C. for 10 minutes, the methylated DNA was resuspended in 30-100 μl H_2O and dissolved at 65° C. for 20 min. Alternatively, methylated DNA was purified using the GeneJET™ gel extraction kit (Fermentas). The success of methylation was checked by specific restriction digests using commercial enzymes such as HaeIII (to confirm methylation by M. CviPI) and HpaII (to confirm methylation by M.SssI).

Conjugation:

For triparental mating, the *E. coli* strains J53 bearing a conjugative RP4 plasmid and HB101 bearing pDAG4 derivatives plus the pRL528 helper plasmid were used (DNA sequence of this plasmid is included as SEQ ID NO. 21, wherein M.AvaI is located between nucleotides 548 to 1996 and M.Eco47II is between nucleotides 3006 to 4259). 3-5 ml of each culture was centrifuged, washed twice with LB medium and suspended in 200 μl of LB-medium. Both *E. coli* strains were mixed, centrifuged and resuspended in 100 μl of LB-medium. 10-15 ml of exponentially growing cyanobac-

terial cultures were centrifuged, washed once with fresh BG11 medium and resuspended in 100-150 µl of BG11. The cyanobacterial and *E. coli* suspension was mixed and applied onto membrane filter (Millipore GVWP, 0.22 µm pore size) placed on the surface of the BG11 medium supplemented with 5% LB in a Petri dish. After incubation under dim light (5 µmol photons/m^2 s) for 2 days, cells were resuspended in fresh BG11 medium, plated onto selective medium (BG11 containing 25-35 µg/ml neomycin) and grown at 30° C. (light intensity approximately 20-40 µmol photons/m^2 s). Sonification and incubation with NaCl (0.5-2M) was not necessary in order to successfully complete the conjugation.

Electroporation:

10-15 ml of a late exponentially growing cyanobacterial culture were centrifuged at room temperature at 3.000×g for 5-10 min. The pellet was washed twice with 0.9% NaCl and then the culture was incubated on ice with 50 mM $CaCl_2$ solution for 15 min. After this step, cells were washed twice with 1.0 mM HEPES pH 7.5, resuspended in 80 µl of 1.0 mM HEPES and chilled on ice. Methylated DNA (0.5-5 µg) was added. Cells were electroporated in a cuvette with a 2-mm gap between the electrodes and pulsed once in a Gene Pulse X-cell (Bio-Rad) using exponential decay protocol (electric field strength 7 kV/cm or 12 kV/cm, capacitance 25 µF; resistance 200 ohms (time constant approximately 5 ms). After electroporation, 2 ml BG11 medium were immediately added to the cyanobacterial suspension, which was subsequently transferred to a 50 ml flask containing 15 ml fresh BG11 medium. After incubation for 2 days under normal light (30-40 µmol photons/2 m^2 s^1) with gentle shaking, recovered cultures were centrifuged, resuspended in 500 µl BG11 medium and placed onto selective media (BG11 containing 25-35 µg/ml neomycin). Cells were incubated under normal light intensity for 4 weeks until colonies were visible.

FIG. **3**A and FIG. **3**B show fluorescence photographs of *Chlorogloeopsis* sp. ABICyano3 cells transformed with an extrachromosomal plasmid harboring the gene encoding the green fluorescent protein under the transcriptional control of a promoter inducible by nitrogen starvation. For induction of the GFP reporter protein, cells were incubated in 1.0 ml of nitrogen-free BG11 medium (BG110) for 2 days. Subsequently, the culture was centrifuged and resuspended in 50-100 µl of BG110 medium. Approximately 10 µl aliquot of the culture was examined by fluorescence imaging microscopy. As negative control an aliquot of wild type culture (in BG11 and BG110 medium) and the non-induced transformant was used. Green fluorescence on a large scale was detected in FIG. **3**B, where the cells were dispersed in nitrogen-free BG110 medium, leading to the induction of the GFP, whereas in FIG. **3**A, less fluorescence was detected in cells suspended in BG11 medium containing nitrogen, due to a basal expression of GFP even in the non-induced state.

Example 5

Determination of Ethanol Production Using Headspace Gas Chromatography with Flame Ionization Detection (GC Online Vial Measurements and GC Single Measurements from Samples Taken from PBR Cultures)

Experimental Setup

Two kinds of GC headspace measurements were performed:

a) GC online vial measurements (applied for clone testing and short-term characterizations of cultures cultivated in GC vials with a duration of up to 72 hours,

b) single GC single measurements (applied for measurements of ethanol concentrations in samples daily taken from PBR cultures) by measuring the ethanol content after transferring 0.5 mL of the PBR cultures into GC vials after certain points of time of cultivation in the PBR.

GC single measurements do not involve the cultivation of the strains in the GC vials. GC single measurements were performed in order to characterize the long term ethanol production of strains, which are already known to produce ethanol in sufficient quantities in GC online vial measurements. GC single measurements further differ from GC online vial measurements in the volume of the culture (2 ml in GC online vial and 0.5 ml aliquots taken from a PBR culture in GC single measurements). In single GC measurements only the absolute amount of ethanol produced at a certain point of time is determined, whereas the GC online vial measurement determines the course of ethanol production during a certain period of time, up to 72 hours of growing the cells in a GC vial under constant illumination. For GC single measurements the sample was heated to 60° C. in order to transfer all ethanol from the liquid phase to the gas phase for the GC headspace chromatography, which resulted in a disruption of the culture. In contrast to that this 60° C. heating step was omitted during GC online vial measurements in order not to destroy the culture and in order to further continue with the culturing of the cells in the GC vial. In the following paragraphs, GC online vial measurements are described.

The GC online vial headspace measurement was performed on a Shimadzu GC-2010 gas chromatograph with Flame Ionization Detector. The detection limit for ethanol quantification is 0.0005%, but a calibration has to be done for detecting quantities below 0.001%. The instrument is connected in-line with a Shimadzu PAL LHS2-SHIM/AOC-5000 autosampler, comprising a gas-tight syringe for transfer of headspace aliquots from the culture samples to the analytical unit. Specific modifications were introduced as follows: Each sample tray was exposed with a LED acrylic sheet (length: 230 mm, wide: 120 mm, diameter: 8 mm, 24Chip, S4, 5300K), equipped with a dimmer by company Sting1 GmbH. Below the sample tray a magnetic stirrer is installed (IKA RO 5 power) allowing for mixing of cultures which are cultivated in GC vials that stand in the sample tray. The sample trays are penetrating of maximum, so that the GC Vial stands in the Tray. A heating mat between an LED acrylic sheet and the magnetic stirrer (MOHR & Co, one heating circuit, 230 V, 200 Watt, length: 250 mm, wide: 150 mm, diameter: ca. 2.5 mm) with a temperature regulator (JUMO dTRON 316) allowed for the incubation of cultures in GC vials at specific temperatures. The gas chromatograph was connected to helium carrier gas as well as hydrogen and artificial air as a fuel gas and an oxidizer gas, respectively, for the flame ionization detector. Oxidizer air was generated with the generator WGAZA50 from Science Support. The gas chromatograph was equipped with a FS-CS-624 medium bore capillary with a length of 30 m, internal diameter of 0.32 mm and film thickness of 1.8 µm from the GC supplier Chromatographie Service GmbH.

The ethanol production in the culture has to be induced 1-2 days before the GC online vial experiment is performed by triggering the overexpression of the PDC enzyme and the ADH enzyme. For induction, hybrid cells grown under repressed conditions in aswBG11 or BG11 freshwater medium (without inductor), were induced when they reached an OD of ~2 by adding the inductor (e.g. metal-ions). The cells were incubated on a small shaker at 180 rpm for 48 hours

at 28° C. The shaker was armed with a dimmable light table adjusted to 120 μE (300 μE-0 μE). After 48 h the tube was centrifuged at 20° C. for 10 minutes, 4,500 rpm, and the supernatant was discarded. The pellet was resuspended in aswBG11 medium or freshwater BG11 medium (in case of PCC9212) suppl. with 50 mM TES pH 7.3, 20 mM $NaHCO_3$, containing inductor (e.g. metal-ions) and no antibiotics. For hybrids under control of copper responsive promoters, the induction was realized by addition of 10-30 μM copper, for zinc inducible promoters the induction was realized by addition of 5-30 μM zinc sulfate (heptahydrate). The sample was adjusted to an OD_{750} of about 0.7 (+/−0.1) for 4 replicates. 2 ml were filled in 20 ml GC vials equipped with a magnetic stir bar (12 mm) in which the lid was not completely tightened. 5 ml pure carbon dioxide was injected for 1-3 days with the 30 ml syringe through the septum, and then the lid tightly closed (gas tight). The tightly closed GC vials were placed into the headspace auto sampler rack which was temperature controlled at a given temperature for example 37° C. and were analyzed at the same day. After the GC measurements the final OD_{750} was determined for the calculation of the ethanol production rate per average OD_{750}. The average OD_{750} was calculated by addition of OD_{750} at t_{start} and OD_{750} at t_{end} divided by two.

When necessary, reference samples for the calibration of the gas chromatograph were prepared as 2 ml aliquots with 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5 and 10 mg/ml ethanol in 35 psu sodium chloride for marine media or without sodium chloride for freshwater media. Reference samples were placed into the same 20 ml sample containers with self-sealing silicon septum caps for headspace autosampling. For each reference sample at least six measurements were applied. After the measurements, the resulting peak areas of the reference samples were used for generating two calibration curves, the first in the concentration range from 0.005 to 0.5 mg/ml ethanol and the second one for the concentration range from 0.5 to 10 mg/ml ethanol. The calibration curves have to fulfill linearity.

The sample incubation temperature for the GC online measurements in the autosampler was adjusted to a given temperature for example 37° C. The illumination was set at 90 μE to 150 μE, preferably 120 μE. The magnetic stirrer was configured for interval mixing of the samples, with cycles of 2 minutes mixing at 400 rpm, followed by 90 minutes without mixing. An automated process follows, wherein after given periods aliquots of 500 μl of the headspace of the samples were automatically drawn with the gas-tight headspace syringe and injected via the injection port into the gas chromatograph for analysis. Before each headspace autosampling, the mixing was changed for 10 min to continuous mixing with 750 rpm at 37° C. incubation temperature. The syringe temperature was set at 70° C. The fill speed was 250 μl per second, following an initial lag time of 1 second after the septum of the samples has been pierced by the syringe needle. The injection of the aliquot into the gas chromatograph happens with an injection speed of 500 μl per second. Afterwards, the syringe flushes for 3 minutes with air to prevent sample carryover between two injections. The gas chromatograph run time was 4 minutes and 30 seconds. The injection temperature on the gas chromatograph was 230° C. The column temperature was 60° C. Detection was accomplished with the flame ionization detector at 250° C. process temperature. The makeup gas was nitrogen at 30 ml per minute, the fuel gas was hydrogen at 35 ml per minute and the oxidizer gas was artificial air at 400 ml per minute.

After the final GC online vial measurement, the final optical density at 750 nm of the samples was measured and an average cell density for each sample was determined by calculating the arithmetic mean of the optical density at the starting point and the optical density at the end point of the process divided by two. Afterwards, the average ethanol production rate per cell density was calculated.

For *Chlorogloeopsis fritschii* PCC6912 and *Chlorogloeopsis* sp. ABICyano3 the above described GC online protocol had to be modified in a way that less stirring/shaking of the agitator (only 250 rpm) was applied. Otherwise a major percentage of the cells aggregated above the liquid surface, subsequently sticking tightly to the wall of the GC vial, leading to an underestimation of the ethanol productivity.

Example 6

Ethanol Production in *Chlorogloeopsis fritschii* PCC6912 and *Chlorogloeopsis* sp. PCC9212 Strains Transformed with Various Plasmids Harboring Ethanologenic Cassettes

Heterologous Promoters:

Chlorogloeopsis fritschii PCC6912 and the more accessible *Chlorogloeopsis* sp. PCC9212 were used for all additional transformation. Methylation of ethanologenic plasmids with M.CviPI followed by electroporation allowed generation of ethanologenic transformants of both *Chlorogloeopsis* strains. The first ethanologenic transformants were generated by use of the methylated plasmids TK18 and TK122. As shown in the FIG. **4**A and FIG. **5**, these plasmids contain the first and second recombinant gene for ethanol production encoding the PDC enzyme and ADH enzyme under the transcriptional control of PpetE from *Nostoc/Anabaena* PCC7120 (TK18) or under the transcriptional control of PziaA from *Synechocystis* PCC6803 (TK122).

Ethanol productivity of *Chlorogloeopsis fritschii* PCC6912 transformed with the plasmid TK18 was measured online in GC vials with high consistency, leading to a production rate of 0.008-0.01% EtOH (v/v)/OD*d in mBG11, whereas the production rate for *Chlorogloeopsis* sp. PCC9212 in BG11 was determined to be 0.01-0.015% EtOH (v/v)/OD*d. FIG. **4**B shows two graphs of the ethanol accumulation during the time course of 18 hours of cultivation measured in GC online experiments with PCC6912 cells transformed with plasmid TK18 with two different starting ODs (graph with squares: start $OD_{750\ nm}$=around 0.5; graph with diamonds: start $OD_{750\ nm}$=around 1). FIG. **4**C shows the accumulation of ethanol in two different cultures of PCC9212 during a cultivation of around 60 hours.

A comparison of the induced *Chlorogloeopsis fritschii* PCC6912 with TK18 to the non-induced control strain transformed with TK18 (data not shown) strongly indicated that PpetE7120 in PCC6912 is not regulated by copper; it appeared to be a constitutive promoter for both *Chlorogloeopsis* sp. strains. Furthermore, due to the constitutive expression of the PDC enzyme and the ADH enzyme, TK18 transformants of both *Chlorogloeopsis* strains grew very slowly during the upscaling due to the constitutive promoter. In addition, loss of productivity was observed after 2-3 weeks of cultivation, due to reversion of the cells to wild type cells.

In contrast to the PpetE promoter from *Nostoc*, the ziaR-PziaA promoter from *Synechocystis* PCC6803 included in the plasmid TK122 was not constitutive, but inducible by Zn^{2+}. Rates of 0.004-0.007% (v/v)/OD*d with TK122 were obtained after one day pre-induction with 10 μM Zn^{2+} followed by GC online measurements with 20 μM Zn^{2+} for *Chlorogloeopsis* PCC9212 cells transformed with this plas-

mid. As the production rate with TK122 was low, the construct was not transformed into *Chlorogloeopsis fritschii* PCC6912.

These data therefore show that the use of heterologous promoters from other cyanobacterial genera in *Chlorogloeopsis* sp. strains leads to either low production rates or to constitutive expression, which can even result in reversion of the genetically enhanced cells back to wild type cells and hence an unstable ethanol production.

Endogenous Promoters of the *Chlorogloeopsis* sp. Strains:

An additional plasmid TK187 was constructed for the *Chlorogloeopsis* sp. strains, which carries the ethanologenic gene cassette under the control of the endogenous putative promoter ziaR-PziaA from *Chlorogloeopsis fritschii* PCC6912 (see FIG. **7**A). The genes ziaR-ziaA (orf5210-5209) were identified by a BLASTP search with the encoded proteins of the *Synechocystis* PCC6803 homologous ziaA and ziaR based on the method described for the additional promoter below. The product of ziaR is a Zn^{2+} dependent transcriptional regulator of ziaA, which represses transcription of ziaA unless Zn^{2+} concentrations are elevated. The product of ziaA encodes a Zn^{2+} transporting ATPase, which transfers zinc tolerance by efficient export of Zn^{2+}.

The putative promoter-regulator combination ziaR-PziaA from *Chlorogloeopsis fritschii* PCC6912 was cloned in two variants: The plasmid TK186 contains a promoter region that covers the -35, -10 box and the first possible ATG start codon of ziaA, while plasmid TK187 contains a longer version, which additionally includes a second downstream located ATG (see FIG. **6** and FIG. **7**A).

Plasmid TK186 which contains the shorter version of the promoter did not result in any ethanol production in both *Chlorogloeopsis* sp. strains PCC6912 and PCC9212, while plasmid TK187 led to a production of up to 0.012-0.015% (v/v)/OD*d in GC online measurements. It is assumed that plasmid TK186 contains a non-functional version of PziaA, due to an incorrect annotation.

FIGS. **7**B to **7**D show graphs of the ethanol accumulation, the activity of ADH and PDC enzyme, the cell growth measured as OD_{750} nm, and the ethanol accumulation in the liquid medium for biological triplicates over 14 days (FIG. **7**B) and a single cultivation for a 7 day cultivation period of *Chlorogloeopsis fritschii* PCC6912 transformed with plasmid TK187 in 0.5 L photobioreactors (Crison).

The culturing conditions in the Crisons were as follows: For upscale, all cultures were maintained under repressed conditions in order to allow a faster upscaling of the cell culture: The cells were scaled up in BG11 under repressed conditions and then transferred to mBG11 medium prepared with artificial seawater salts (35 psu) and deionized water. The medium was supplemented with Vitamins B1 and B12. Neomycin (100 mg L^{-1}) was used for plasmid retainment. Induction was initiated by the addition of 10 μM Zn^{2+}. It is beneficial to use a sufficiently high initial cell density for *Chlorogloeopsis fritschii* PCC6912. Otherwise, the culture behaves highly susceptible to light stress (caused by a high light regime or high mixing). For the standard set up, an initial OD_{750} of about 2 proved well suited. Cells were cultivated in 0.5 L round Crison bottles. Mixing was achieved with a magnetic stir cross (250-450 rpm) and applied for 24 h. Cultures were run at pH 8.0. The pH was maintained by injection of 10-20% CO_2 in air into the liquid phase. The total gas flow rate was 15 ml min^{-1} (applied only for pH control). There was no aeration or pH control during the night. A light: dark photoperiod of 12 h:12 h was applied. Illumination of cultures was done with LED lamps (which illuminate the round bottle from all sides). At the beginning of cultivation a light intensity of ca. 325-450 μmol s^{-1} m^{-2} was applied. Later during cultivation light was increased to 900 μmol s^{-1} m^{-2}. The temperature regime was set to 25-28° C. during the night and 35-38° C. during the day. In all cultures, $OD_{750\ nm}$, chlorophyll and ethanol (as well as acetaldehyde) content were analyzed at least three times a week. PDC and ADH activities were recorded twice a week. Absorption spectra of cells were recorded weekly. Data on culture temperature and pH as well as oxygen saturation were automatically recorded online via probes.

For the biological triplicates of hybrid PCC6912 with TK187 a mean productivity of 0.0327% (v/v) d^{-1} could be achieved for 14 days (see FIG. **7**B). Furthermore, for a short time frame: of 7 day production period, a mean rate of 0.0398% (v/v) d^{-1} was achieved for biological triplicates while another single cultivation resulted in a 7 day rate of 0.0452% (v/v) d^{-1} (see FIG. **7**D).

FIG. **7**E shows the accumulation of ethanol (% (v/v)) during a nearly 70 hour cultivation of *Chlorogloeopsis* sp. PCC9212 transformed with the plasmid TK187 determined via GC online measurements. It is clearly visible that with an increasing degree of induction of the cells with Zn^{2+} (5, 10 or 30 μM Zn^{2+} denoted as 5Zn, 10Zn or 30Zn), higher ethanol accumulation can be achieved, leading to ethanol production rates of up to 0.0122% (v/v)/OD*d.

FIG. **8** denotes the plasmid map of the plasmid TK261 wherein the same regulator-promoter pair ziaR-PziaA as included in plasmid TK187 only controls the transcription of the first recombinant gene encoding PDC enzyme, whereas the constitutive promoter PrbcL* controls the transcription of the second recombinant gene coding for synADH enzyme. *Chlorogloeopsis fritschii* PCC6912 cells harboring this plasmid and cultivated in the 0.5 L Crison-photobioreactors achieved an ethanol production rate of 0.02%(v/v) d^{-1} in the liquid phase over a period of 14 days, see FIG. **18** (conditions as follows: Media: mBG11 (35 psu (ASW) Neo100; Cultivation pH: 8.0; CO_2 supply: 10% pH dependent into liquid phase; Aeration: 10 mL min-1; Mixing: 250 rpm magnetic cross bar (comet); Light: fluorescence lamps starting with 275 μmol s^{-1} m^{-2}, later increased to 450 from two sides 12/12 h).

Further derivatives of the initially successful plasmid TK187 were developed. The plasmid map of such a derivative TK336, (see FIG. **9**A) shows its differences in comparison to TK187. In particular, the regulator-promoter pair ziaR-PziaA controls the transcription of a codon improved variant of the first recombinant gene coding for PDC enzyme. The second recombinant gene is also codon improved and is transcriptionally controlled by PnblA from *Nostoc/Anabaena* PCC7120, which is a constitutive promoter in the *Chlorogloeopsis* PCC6912 and PCC9212 host cells. In addition the transcriptional control of both recombinant genes for ethanol production is decoupled via an oop terminator present between both genes. FIG. **9**B shows the ethanol accumulation of cultures of *Chlorogloeopsis fritschii* PCC6912 with TK336 as determined by GC online measurements without induction (graph denoted —Zn) and with induction by 30 μM Zn^{2+} (graph denoted 30Zn) leading to a production rate of 0.015-0.02% (v/v)/OD*d. A rise in the accumulation of ethanol was clearly visible upon induction of PziaA. *Chlorogloeopsis* PCC6912 cells transformed with this plasmid and cultivated in the 0.5 L Crison-photobioreactors achieved an ethanol production rate of 0.022% (v/v) d^{-1} in the liquid phase for 14 days (conditions as mentioned above for TK261). The cultivation of *Chlorogloeopsis* PCC 6912 cells transformed with TK336 in 0.5 L Crison-photobioreactors is shown in FIG. **18**.

FIG. **10**A shows the plasmid map of the plasmid TK414 including the Zn^{2+} inducible promoter PziaA from *Chlorogloeopsis* PCC6912 controlling the transcription of codon improved variants of pdc and adh genes. This plasmid is a derivative of the initially successfully transformed plasmid. In addition, a terminator sequence (oop terminator) is located downstream of the *Synechocystis* ADH enzyme encoding gene in order to ensure a reliable transcription termination.

FIG. **10**B includes a graph depicting a comparison of the ethanol accumulation (% (v/v) of *Chlorogloeopsis* PCC6912 hybrid cultures containing the different plasmids TK414 and TK187 during 15 day 0.5 liter photobioreactor cultivations determined via GC single measurements. The accumulation of ethanol in both cultures was very similar, indicating that TK414 results in slightly higher ethanol productivity, especially after 14 days of cultivation (and could be slightly advantageous with regard to long term production). The cultivation conditions were as follows: BG11, 250 rpm, light: fluorescence lamps starting with 2×160 μmol s^{-1} m^{-2}, later increased to 2×360 μmol s^{-1} m^{-2}, mixing: 250 rpm (no vitamins added).

Additional Inducible Endogenous Promoters of *Chlorogloeopsis fritschii* PCC6912 in Addition to PziaA:

In order to initially identify PziaA and other additional inducible promoters which could lead to higher ethanol productivity rates, the genome of *Chlorogloeopsis fritschii* PCC6912 cells was searched for genes encoding metal ion transporters and metallothioneins, respectively. ORFs were chosen by:

a) the degree of similarity to ZiaA and SmtA

b) the genetic organization reflecting the adjacent localization of ziaR-ziaA.

11 putative genes plus a potential ziaA homolog, whose promoter is cloned in TK187, were selected and primers for qRT-PCR were designed. Cultures were treated with a metal mix containing 20 μM Co^{2+}, 30 μM Zn^{2+} and 1 μM Cu^{2+}.

Total RNA from PCC6912 treated with the metal mix and from a control culture grown in BG11 medium and in BG11 medium without Co^{2+}, Zn^{2+} and Cu^{2+} (traces for preparation of BG11 medium were prepared without Co^{2+}, Zn^{2+} and Cu^{2+}) were isolated and Quantitative reverse transcription PCR (qRT-PCR) was performed to analyze which of the 11 putative genes including ziaA respond upon induction with the metal ions in higher gene expression. In particular the following procedure was employed:

To remove traces of DNA, two DNase steps were applied. First an on-column digest according to Qiagen was performed, followed by a DNaseI (Roche) incubation for 1-2 h at 37° C. Success of DNase treatment was controlled using the primers T394 and T395 (the DNA sequences of both primers are included in the sequence listing as SEQ ID NO. 24 and 25, respectively) against the gapA reference gene. 1 μg DNase-free RNA was transcribed into cDNA using the QuantiTect Rev. Transcription Kit from Qiagen. Quantitative RT-PCR was performed in triplicates with RNA from three independent preparations using a LightCycler 480 (Roche) and the Roche LightCycler 480 SYBR Green I Master. Gene specific-primers used for qRT-PCR amplification (the qRT-PCR, orf7041 forward and reverse primers are shown as SEQ ID NOs. 26 and 27, the qRT-PCR, orf5189 forward and reverse primers are shown as SEQ ID NOs. 28 and 29, the orf7345 forward and reverse primers are shown as SEQ D NOs. 30 and 31, the qRT-PCR, orf5209 (ziaA) forward and reverse primers are SEQ ID NOs. 32 and 33, and orf5203 forward and reverse primers are shown as SEQ ID NOs. 34 and 35, respectively) were designed to produce a 120-150 bp amplicon. The amount of PCR product was quantified by measuring fluorescence of SYBR Green dye. Reported gene expression levels were normalized to levels of the gapA gene.

FIG. **11**A shows the metal-ion dependent induction of orf7041 by qRT-PCR. PCC6912 was grown for 48 h in BG11 medium (C1, C2, C3), in BG11 medium without Co^{2+}, Zn^{2+}, Cu^{2+} (C4, C5, C6) and in BG11 medium containing 20 μM Co^{2+}, 30 μM Zn^{2+}, and 1 μM Cu^{2+} (C7, C8, C9). cDNA was prepared from cultures under all treatments. Gene specific-primers used for qPCR amplification are included in the sequence listing as SEQ ID 24-27. The amount of the PCR product was quantified by measuring fluorescence of the SYBR Green dye (y-axis) over the number of PCR cycles (x-axis). The fluorescence increases proportionally to the number of amplified fragments and can be measured when the fluorescence significantly rises above the background fluorescence. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. In this case, higher copy number of orf7041 from cultures treated with 20 μM Co^{2+}, 30 μM Zn^{2+}, and 1 μM Cu^{2+} (C7, C8, C9) are observed compared to copy numbers from cultures grown in BG11 or BG11 medium without metal ions (C1-C6). qRT-PCR shows the significant upregulation of orf7041 by addition of the metal-ion mix containing 20 μM Co^{2+}, 30 μM Zn^{2+}, and 1 μM Cu^{2+}. The promoter of orf7041 can be considered as being regulated by at least one of these metal-ions.

FIG. **11**B shows the 484 fold upregulation of orf7041 by metal-ions. Based on the amplification curves shown in FIG. **11**A, relative quantification was performed by the light cycler 480 software (Roche). For this purpose, ratios of the target (orf7041) and reference (gapA) expression levels were calculated (light blue bars) for each growth condition i) BG11 (left bars), ii) without Co^{2+}, Zn^{2+}, Cu^{2+} (middle bars), iii) plus 20 μM Co^{2+}, 30 μM Zn^{2+} and 1 μM Cu^{2+} (right bars). orf7041 expression ratios were further normalized to expression levels of PCC6912 grown in BG11 medium (red bars). Repression of orf7041 transcription was observed in PCC6912 grown without Co^{2+}, Zn^{2+} or Cu^{2+} (middle bars) whereas the mixture of 20 μM Co^{2+}, 30 μM Zn^{2+} and 1 μM Cu^{2+} induced the expression 484-fold. Primers for gap and orf7041 are included in the sequence listing as SEQ ID 24-27.

From the shown qRT-PCR results, the promoter of orf7041 can therefore be considered as regulated by at least one of these (Cu^{2+}, Co^{2+}, Zn^{2+}) metal-ions. Later experiments demonstrated Co^{2+} as being the best inductor for Porf7041.

Promoters of those genes, which were found to be regulated upon induction with the metal ions, were cloned.

In the following, some of the plasmids including these endogenous promoters and experimental data obtained by introducing these plasmids into the *Chlorogloeopsis* sp. host cells will be described in more detail.

The plasmid map of TK346 is shown in FIG. **12**A including the promoter of the open reading frame (orf) 7041 from *Chlorogloeopsis* PCC6912 which is a cobalt inducible promoter, controlling the transcription of a codon improved version of the first recombinant gene encoding PDC enzyme. The *Synechocystis* ADH enzyme encoding second recombinant gene is controlled by the constitutive promoter PnblA from *Nostoc* and a transcription terminator sequence (oop terminator) is present between both recombinant genes in order to decouple the transcriptional control of these genes. For *Chlorogloeopsis* PCC6912 cells harboring this plasmid ethanol production rates of about 0.012% (v/v)/OD*d could be determined after induction with 30 μM Co^{2+}. FIG. **12**B shows a graph evidencing the ethanol accumulation over a 20 hour cultivation of an induced culture of this strain determined via GC online measurements. FIG. **12**C depicts the

ethanol accumulation over the time course of 12 days of these cultures in larger 0.5 liter photobioreactors determined via GC single measurements. FIG. **18** shows a comparison of PCC6912 hybrids containing either the Co^{2+} inducible plasmid/promoter TK346 or one of the PziaA containing plasmids (TK261, TK336). Under the cultivation conditions used for the cultivation shown in FIG. **18**, it can be seen that the ethanol yield of TK346, with a production rate of 0.025% (v/v) d^{-1} over 14 days and 0.02% (v/v) d^{-1} over 18 days (from day 2-20) was even slightly better than the PziaA constructs.

FIG. **13** shows the plasmid map of the plasmid TK348 including the promoter controlling the open reading frame (orf) 7345 of *Chlorogloeopsis* PCC6912, which is primarily a Zn^{2+} but also a Co^{2+} inducible promoter and which controls the transcription of both the PDC enzyme and the *Synechocystis* ADH enzyme encoding first and second recombinant genes.

FIG. **14** shows the plasmid map of the plasmid TK351 including the Zn^{2+} inducible promoter of the open reading frame (orf) 5189 of *Chlorogloeopsis* PCC6912 controlling the transcription of both the PDC enzyme and *Synechocystis* Adh enzyme encoding genes. *Chlorogloeopsis* PCC6912 cells including this plasmid achieved an ethanol production rate of around 0.008% (v/v)/OD*d upon induction in GC vials.

FIG. **15** shows the plasmid map of the plasmid TK380, including the Zn^{2+} inducible promoter of the open reading frame (orf) 5203 of *Chlorogloeopsis* PCC6912, controlling the transcription of both the first and second recombinant gene encoding the PDC and ADH enzyme.

The above described procedure can be used to identify further endogenous metal ion inducible promoters in the *Chlorogloeopsis* sp. host cells.

Example 7

Transformation of *Chlorogloeopsis* sp. Host Cells Using Integrative Plasmids

In the following, the design of three different constructs will be discussed, which were prepared in order to transform *Chlorogloeopsis* sp. host cells in the future by integrating genes into their genomes.

In particular, integration of a resistance marker into the genome of the *Chlorogloeopsis* sp. host cells, such as *Chlorogloeopsis fritschii* PCC6912 is conducted with the help of plasmids TK148, TK149, (see FIG. **16**A and FIG. **16**B for plasmid maps of both constructs) which were generated to integrate a neomycin/kanamycin resistance gene into orf1237 (pilA) or orf3194 (blpI) of the *Chlorogloeopsis* sp. host cells via homologous recombination, respectively. In general, the integrative plasmids contain two platforms (homologous sequence regions) for homologous recombination into the *Chlorogloeopsis* sp. host cells, which flank a DNA sequence to be introduced into the genome of the cyanobacteria. These plasmids are based on the cloning vector pGEM and contain flanking regions (down and upstream) of orf1237/orf3194 upstream and downstream of the neomycin/kanamycin resistance gene to generate a double crossover event in the *Chlorogloeopsis* sp. host cells.

Integration of other target genes, such as recombinant genes for ethanol production, into the genome of the *Chlorogloeopsis* sp. host cells is achieved with the integrative plasmid TK153 (see FIG. **16**C for the plasmid map of this vector), which was generated to integrate a PnblA7120-PDC-synADH cassette as well as a neomycin/kanamycin resistance gene into orf1237 (pilA). The pGEM based plasmid contains a flanking region of orf1237 of PCC6912 upstream of PnblA7120-PDC-synADH as well as a flanking region of orf1237 of PCC6912 downstream of the neomycin/kanamycin resistance gene. TK153 was designed to generate a double crossover event via homologous recombination in PCC6912 in order to insert PnblA7120-PDC-synADH as well as a neomycin/kanamycin resistance gene into the genome of PCC6912. The promoter PnblA from *Nostoc/Anabaena* PCC7120 is a constitutive promoter in PCC6912 and PCC9212.

In general, the transformation using integrative plasmids could be done in the same way as the transformation using the extrachromosomal plasmids.

Example 8

Salt- and Freshwater Cultivation of *Chlorogloeopsis* PCC6912 Cells Transformed with Plasmid TK336

Since *Chlorogloeopsis* PCC6912 was isolated from a freshwater habitat, its ability to grow and produce ethanol under saltwater conditions is of great importance. The use of saltwater as a culture medium would greatly ease the cultivation and would reduce cultivation costs in comparison to cultivation in fresh water medium.

For the cultivations shown in FIGS. **17**A and **17**B, *Chlorogloeopsis* PCC6912 cells transformed with the plasmid TK336 where slowly adapted to the salt concentrations. From solid BG-11 plates, cells were directly inoculated in liquid BG-11, without Zn^{2+} (repressed conditions). After two days, the cells were transferred to artificial seawater media with 8.75 psu and 17.5 psu ASW BG-11 and were set to an identical $OD_{750\,nm}$. For the higher salt concentrations, cells grown in 17.5 psu were transferred to media with 26.25 psu and 35 psu and were set to an identical $OD_{750\,nm}$. Cells were kept under these conditions for one more week and subjected to relatively low illumination intensities from two fluorescence lamps with 125 μmol s^{-1} m^{-2} each. Furthermore, no vitamins were added to the culture medium. For plasmid retainment and contamination control, kanamycin was used, adjusted to the salt concentration, as well. 8.75 psu kanamycin 37.5 mg/L, 17.5 psu kanamycin 50 mg/L, 26.25 psu kanamycin 100 mg/L.

FIG. **17**A shows the ethanol accumulation over a course of 22 days in *Chlorogloeopsis* PCC6912 cells containing the plasmid TK336 growing in medium with different salinities of 8.75, 17.5, 26.25 and 35 psu. This graph evidences that the ethanol accumulation was the highest at lower salinities, e.g. between salinities of 8.75 to 17.5 psu, which shows that *Chlorogloeopsis* PCC6912 cells, although isolated from freshwater, can grow and produce ethanol in brackish water, although ethanol production in freshwater medium was higher compared to brackish medium.

FIG. **17**B shows a comparison of the ethanol production rate (% (v/v) d^{-1}) between days 5 to 12 and days 5 to 23 for the same cells already shown in FIG. **17**A at different salinities measured via GC single measurements. Again it can be seen that the ethanol production rate was the highest for salinities between of 8.75 to 17.5 psu.

FIG. **18** shows the ethanol accumulation in 35 psu medium (artificial mBG11) at pH 8 of parallel cultivations of *Chlorogloeopsis* PCC6912 cells harboring the plasmids TK261, TK336 (Zn^{2+} inducible promoter regulator pair PziaA-ziaR controlling pdc gene in both plasmids TK261 and TK336) and TK346 (Porf7041 controlling pdc gene), respectively.

Cultivations were done in 0.5 L Crison photobioreactors with stirring at 250 rpm. The cells were subjected to an illumination intensity of 2×275 μmol s^{-1} m^{-2}, (275 μmol s^{-1} m^{-2} from two different sides of the photobioreactor) increasing to 2×450 μmol s^{-1} m^{-2}. The induction was initiated by adding 10 μM Zn^{2+} and 10 μM Co^{2+} (TK346), respectively. This graph clearly shows that during a 20 day cultivation, ethanol accumulation values of between 0.3 to 0.4% (v/v) could be reached, which was significantly higher compared to the experiments shown in the FIG. **17**A and FIG. **17**B in 35 psu BG11 medium.

Although the present invention has been described in considerable detail with reference to certain embodiments thereof, other embodiments are possible. Therefore, the spirit and scope of the appended claims should not be limited to the description of the embodiments contained therein.

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<211> LENGTH: 373
<212> TYPE: PRT
<213> ORGANISM: Anabaena PCC7119

<400> SEQUENCE: 15

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Val His Thr Arg Ile Gly Arg Glu Pro Cys Ala Arg Leu Trp Tyr Leu
35 40 45

Cys Arg Ala Leu Asp Lys Asp Gly Ser Gly His Leu Thr Leu Pro Leu
50 55 60

Pro Val Val Gln Thr Phe Leu Asp Cys Ser Asp Lys Ser Val Tyr Arg
65 70 75 80

Trp Leu Gln Asp Gly Lys Lys Ile Gly Ala Phe Arg Arg Tyr Lys Ile
85 90 95

Lys Ala Gly Met Ile Thr Val Tyr Leu Gly Gly Met Phe Gln Val Cys
100 105 110

Tyr Asn Leu Asn Leu Lys Arg Trp Gly Asp Val Ala Val Val Pro Leu
115 120 125

Val Gln Val Leu Ser Asp Leu Arg Ser Leu Thr Thr Gly Ile Val Thr
130 135 140

Gln Ser Phe Gln Gln Lys Ser Arg Tyr Ala Ala Asn Arg Gln Leu Lys
145 150 155 160

Pro Glu Tyr Arg Lys Leu Phe Gly Ala Pro His Pro Asn Glu Leu Val
165 170 175

Lys Asp Thr Arg Gln Ser Ser Leu Lys Ser Pro Glu Gly Glu Val Pro
180 185 190

Cys Val Leu His Ile Ser Ser Ser Arg Ile Phe Val Ser Lys Ser Phe
195 200 205

Ile His Tyr Gly Thr Ser Gln Lys Ala Val Ser Cys Glu Leu Gly Ile
210 215 220

His Lys Arg Thr Val Arg Arg His Gln Lys Gln Leu Gly Met Asn Arg
225 230 235 240

Arg Gln Leu Cys Gln Ala Lys Ile Glu Tyr Asn Gln Leu Arg His Ala
245 250 255

Arg Asn Asn Asp Ala Ser Glu Phe Trp Ala Phe Thr Gly Thr Lys Thr

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Asp Ile Gly Tyr Gln Val Met Gly Asp Ala Val Val Phe Ser Asp Gly
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Ile Ala Pro Gly Ala Lys Lys Arg Gln Pro Asn Thr Tyr Gln Ile Asp
    290                 295                 300

Ala Thr Glu Phe Asp Gly Arg Leu Phe Lys Val Gly Asp Lys Val Phe
305                 310                 315                 320

Met Asn Arg Cys Asn Ile Tyr Arg Glu Gln Phe Thr Leu Thr Thr Met
                325                 330                 335

Ser Ala Ala Arg Arg Lys Tyr His Phe Lys Leu Ser Gln Cys His Phe
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Ser Glu Asn Arg Ala Gly Arg Val Gly Asn Arg Phe Val Ile Gly Cys
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His Ser Gly Glu Ile
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<210> SEQ ID NO 16
<211> LENGTH: 183
<212> TYPE: PRT
<213> ORGANISM: Anabaena PCC7119

<400> SEQUENCE: 16

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Leu Phe Gly Ile Cys Leu Phe Thr Gly Cys Arg Val Ser Glu Ala Leu
        35                  40                  45

Ala Leu Gln Thr Thr Asp Ile Lys Gly Glu Thr Leu Thr Phe Arg Lys
    50                  55                  60

Ser Thr Thr Lys Gly Lys Leu Lys Thr Arg Val Val Asp Ile Gln Pro
65                  70                  75                  80

Gly Leu Ala Ala Leu Met Ala Asp Tyr His Pro Lys Pro Gly Thr Leu
                85                  90                  95

Phe Pro Gly Met Arg Gly Val Ser Asp Arg Leu Thr Arg Tyr Ala Ala
            100                 105                 110

Asp Lys Ile Leu Arg Asp Ala Ala Lys Arg Ile Gly Leu Glu Gly Ile
        115                 120                 125

Ser Thr His Ser Phe Arg Arg Thr Ala Leu Asn Gln Met Ser Ser Ala
    130                 135                 140

Gly Ile Pro Leu Arg His Ile Gln Glu Ile Ser Gly His Asn Asp Leu
145                 150                 155                 160

Gly Thr Leu Gln Arg Tyr Leu Glu Val Thr Pro Glu Gln Arg Arg Lys
                165                 170                 175

Ala Val Ser Val Ile Gly Phe
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<210> SEQ ID NO 17
<211> LENGTH: 3718
<212> TYPE: DNA
<213> ORGANISM: Anabaena PCC7119

<400> SEQUENCE: 17

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aaaagttcag tgcttggagg ttgttatgct acttaaccga agctaaataa aacacacata 480

cacagaattg ttaccacacc aaacaccaaa cagaaaaaac gtgctttgaa tttatcaatt 540

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<210> SEQ ID NO 18
<211> LENGTH: 13729
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: plasmid K230

<400> SEQUENCE: 18

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We claim:

1. A genetically enhanced *Chlorogloeopsis* sp. host cell comprising at least one first recombinant gene encoding a first protein for the production of ethanol under the transcriptional control of a first inducible promoter, wherein said first inducible promoter has at least 85% sequence identity to nucleotides 62-87, 101-106, and 133-139 of SEQ ID NO: 36.

2. The genetically enhanced *Chlorogloeopsis* sp. host cell of claim **1**, wherein the host cell is *Chlorogloeopsis fritschii* PCC6912, *Chlorogloeopsis* sp. PCC9212, or *Chlorogloeopsis* sp. ABICyano3ATCC #PTA-120619).

3. The genetically enhanced *Chlorogloeopsis* sp. host cell of claim **2**, wherein the host cell is *Chlorogloeopsis fritschii* PCC6912.

4. The genetically enhanced *Chlorogloeopsis* sp. host cell of claim **1**, further comprising at least one second recombinant gene encoding a second protein for the production of ethanol.

5. The genetically enhanced *Chlorogloeopsis* sp. host cell of claim **1**, wherein the first recombinant gene encodes pyruvate decarboxylase.

6. The genetically enhanced *Chlorogloeopsis* sp. host cell of claim **4**, wherein the second recombinant gene encodes alcohol dehydrogenase.

7. The genetically enhanced *Chlorogloeopsis* sp. host cell of claim **1**, wherein the first recombinant gene encodes alcohol dehydrogenase E (AdhE) converting Acetyl-CoA into ethanol.

8. The genetically enhanced *Chlorogloeopsis* sp. host cell of claim **4**, wherein both the first and second recombinant gene are under the transcriptional control of the same first endogenous inducible promoter.

9. The genetically enhanced *Chlorogloeopsis* sp. host cell of claim **4**, wherein the first and second recombinant genes are under the transcriptional control of separate first and second promoters.

10. The genetically enhanced *Chlorogloeopsis* sp. host cell of claim **9**, wherein the second promoter is a constitutive promoter.

11. The genetically enhanced *Chlorogloeopsis* sp. host cell of claim **9**, wherein the second promoter is an inducible promoter.

12. The genetically enhanced *Chlorogloeopsis* sp. host cell of claim **4**, wherein the second promoter is a constitutive promoter selected from a group consisting of PpetE, PnblA from *Nostoc*.

13. The genetically enhanced *Chlorogloeopsis* sp. host cell of claim **4**, wherein the at least one first and/or second recombinant gene is codon improved for enhancing translation by having a codon adaptation index (CAI) of ≥0.60.

14. The genetically enhanced *Chlorogloeopsis* sp. host cell of claim **4**, wherein a transcription terminator is present between the first and second recombinant gene.

15. The genetically enhanced *Chlorogloeopsis* sp. host cell of claim **4**, wherein the at least one first recombinant gene and at least one second recombinant gene are located on an extrachromosomal plasmid of the host cell.

16. The genetically enhanced *Chlorogloeopsis* sp. host cell of claim **13**, wherein the extrachromosomal plasmid contains an origin of replication which is at least 90% identical to the origin of replication of the pDU1 plasmid.

17. The genetically enhanced *Chlorogloeopsis* sp. host cell of claim **4**, wherein the at least one first recombinant gene and at least one second recombinant gene are integrated into a chromosome of the host cell.

18. The genetically enhanced *Chlorogloeopsis* sp. host cell of claim **1**, which can withstand at least one of the following culturing conditions:

a) At least 1% (v/v) ethanol in the medium for at least 6, 12, 16 or 27 weeks,

b) at least 48° C. for at least 2 hours peaks over at least 7 days in brackish medium, and

c) purging with 60% (v/v) to 70% oxygen.

19. A construct for transformation of *Chlorogloeopsis* sp. host cells comprising:

at least one first recombinant gene encoding a first protein for the production of ethanol under the transcriptional control of a first inducible promoter, wherein said first inducible promoter has at least 85% sequence identity to nucleotides 62-87, 101-106, and 133-139 of SEQ ID NO: 36.

* * * * *